Short Communication

Novel spectroscopic aspects of Type I copper in *Hyphomicrobium* nitrite reductase

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Abstract

Copper-containing nitrite reductase isolated from *Hyphomicrobium* sp. A 3151 has been characterized by electronic absorption, circular dichroism (CD) and electron paramagnetic resonance (EPR) spectroscopies. The visible absorption spectrum of Type I copper (blue copper) in the enzyme especially indicates novel features compared with those of Type I coppers in not only several nitrite reductases already reported but also small blue copper proteins. The EPR spectrum of Type I copper exhibits an axial symmetry.

Introduction

Nitrite reductases play a role in the reduction of nitrite to nitric oxide during the course of denitrification, which is the dissimilatory reduction of nitrate or nitrite to produce molecular nitrogen in prokaryotic organisms. The copper-containing nitrite reductases from *Achromobacter cycloclastes* IAM 1013 [1, 2], *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* [3], *Alcaligenes faecalis* strain S-6 [4] and *Bacillus halodenitrificans* [5] have been known to contain two types of copper (Type I (blue) and Type II (non-blue) coppers), whereas the enzymes from *Achromobacter xylosoxidans* NCIB 11015 [6] and *Pseudomonas aureofaciens* [7] have been reported to contain blue copper only.

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The three-dimensional crystal structure of nitrite reductase isolated from *Achromobacter cycloclastes* IAM 1013 has been recently determined to 2.3 angstrom resolution [8]: the enzyme is a trimer (about 105 kDa) and two copper atoms in the monomer comprise one Type I copper site (two His, one Cys and one Met ligands) and one Type II copper site (three His and one solvent ligands).

Hyphomicrobium sp. A3151 utilizes methanol as an electron donor in the course of denitrification. In the present paper, we describe the unique spectroscopic aspects of Type I copper in the new nitrite reductase.

Experimental

Hyphomicrobium sp. A3151 cells were grown at 30 °C for 5 days under static conditions in a thick liquid phase of a mineral salts medium containing 1% (vol./vol.) methanol and 1% (wt./vol.) potassium nitrate [9, 10].

The isolation and preparation of the enzyme is as follows [10]. The cells suspended in 0.01 M phosphate buffer (pH 7.0) were sonicated, followed by ultracentrifugation (105 000 × g) for 1 h. Ammonium sulfate was added to the supernatant solution to give 40% (wt./vol.) concentration, and the precipitate containing crude nitrite reductase was collected by centrifugation. The precipitate was dissolved in distilled water and the solution was applied to a Sephadex G-200 column equilibrated with 0.01 M phosphate buffer (pH 7.0). The blue fraction was eluted with the same buffer and purified with DEAE-Sephadex, Sephadex G-150 and hydroxyapatite columns. SDS-polyacrylamide gel electrophoresis of this sample showed a single band (molecular weight, 49 kDa) by protein staining. Since the molecular weight of the enzyme was determined to be 200 kDa by gel filtration using Sephadex G-150, *Hyphomicrobium* nitrite reductase is considered to be composed of four identical subunits. The copper content was estimated to be three atoms per one subunit by use of a Seiko atomic absorption spectrometer SAS-760. Electronic absorption and CD spectra were recorded at room temperature with a Shimadzu MPS 2000 spectrophotometer and a JASCO J-500A spectropolarimeter, respectively. EPR spectra at 77 K were taken with a JEOL JES-FE1X spectrometer with 100 kHz field modulation. The enzyme activity of the purified enzyme was estimated according to the method of Kakutani *et al.* [4].

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Results and discussion

The electronic absorption and CD spectra of *Hyphomicrobium* nitrite reductase in 0.1 M phosphate buffer (pH 7.0) are shown in Fig. 1(a) and (b), respectively. The absorption spectrum displays three peaks at 277, 456 and 605 nm. The intense band around 280 nm is assigned to the electronic transitions of aromatic amino acid residues in the protein, and the 456 and 605 nm bands are predominantly attributed to a Type I copper (blue copper) chromophore [11]. There are two spectroscopically unique characteristics of this enzyme. First, the molar absorptivity ratio ($\epsilon_{280}/\epsilon_{600} = 11\,000/2\,600 = 4.2$) of the protein band around 280 nm to the so-called blue band around 600 nm is considerably smaller than those of copper-containing nitrite reductases already reported: *A. cycloclastes* IAM 1013: $\epsilon_{280}/\epsilon_{600} = 58\,000/2\,000 = 29$ [1] and 22 [2]; *R. sphaeroides* forma sp. *denitrificans*: 62 000/6 200 = 10 [3]; *A. faecalis* strain S-6: 117 000/5 400 = 22 [4]; *A. xylosoxidans* NCIB 11015: 54 000/3 700 = 15 [6]; *P. aureofaciens*: 91 000/7 000 = 13 [7]; *B. halodenitrificans*: 88 800/4 900 = 18 [5]. The small value is similar to those of electron transfer proteins containing a blue copper such as stellacyanin (5.7) [12] and umecyanin (3.7) [13], whereas the corresponding values of azurin and plastocyanin are less than 3 [14–16]. Second, the visible spectrum exhibiting two absorption maxima at 456 and 605 nm and a shoulder band around 800 nm is quite unique in comparison with the spectra of the above six copper-containing nitrite reductases [5]. Especially the so-called blue band at 605 nm, which is due to an S(Cys) → Cu charge transfer transition [11, 17], is shifted to longer wavelengths compared to the corresponding bands (587–595 nm) of six nitrite reductases [5]. This absorption maximum resembles those of stellacyanin (604 nm) [12] and cucumber ascorbate oxidase (607 nm) [18]. Moreover, the 456 nm band assigned to both N(His) → Cu and S(Met) → Cu charge transfer transitions [11, 17] shows a large molar absorptivity ($\epsilon = 1\,500$ M^{-1} cm^{-1}) and the $\epsilon_{456}/\epsilon_{605}$ ratio is estimated to be 0.58. On the other hand, the corresponding ratio ($\epsilon_{448}/\epsilon_{604}$) of stellacyanin is 0.15 [12] and the absorption spectrum of cucumber ascorbate oxidase does not have the absorption maximum around 450 nm [18].

In the CD spectrum of the oxidized enzyme (Fig. 1(b)), four extrema appeared in the visible region, 400 (+), 460 (−), 570 (+) and 695 (−) nm. The typical CD pattern of the Type I copper chromophore is more similar to that of amicyanin (a blue copper protein) from *Pseudomonas* sp. strain AM1 (385 (+), 460 (−), 580 (+) and 690 (−) nm) than those of stellacyanin [12] and cucumber ascorbate oxidase [16], although amicyanin shows the blue band at 596 nm [19].

In conclusion, the unique absorption and CD spectra of *Hyphomicrobium* nitrite reductase imply that the geometry of the Type I cupric center might be different from those of Type I copper centers already reported, although the ligating groups consist of an N_2SS^* (N, imidazole group of His; S, thiol group of Cys; S*, thioether group of Met) like many Type I copper centers.

Figure 2 shows the EPR spectrum of *Hyphomicrobium* nitrite reductase at 77 K. The spectrum indicates the existence of two kinds of copper: major Type I copper with EPR parameters of $g_{//}$ 2.24, $g_{\perp}$ 2.07 and $A_{//}$ 5.5

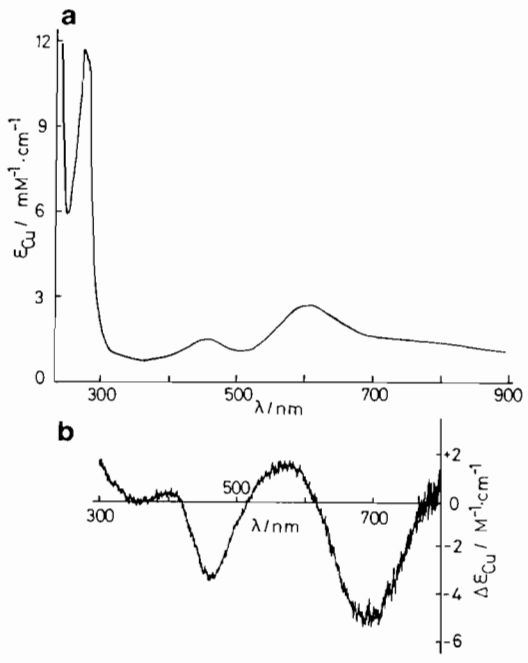

Fig. 1. Electronic absorption (a) and CD (b) spectra of *Hyphomicrobium* nitrite reductase in 0.1 M potassium phosphate buffer (pH 7.0) at room temperature.

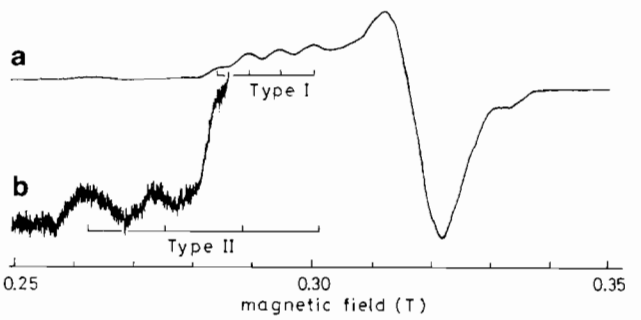

Fig. 2. X-band EPR spectrum of *Hyphomicrobium* nitrite reductase in 0.1 M potassium phosphate buffer (pH 7.0) at 77 K. (a) Modulation = 6.3 G; relative signal gain = 1. (b) Modulation = 6.3 G; relative signal gain = 10.

mT and minor Type II copper with those of g_z 2.34 and A_z 11.5 mT. The weak signal around 0.335 T might belong to the Type II copper with part of the hyperfine lines in the region 0.26–0.28 T. The EPR spectrum of the Type I copper center exhibits an axial symmetry like plastocyanin and azurin, where the copper center has two histidine imidazoles and two sulfur atoms from Met and Cys residues as ligands. The intensity of the Type II copper signal between 0.26 and 0.28 T is much weaker than that of the hyperfine lines of the Type I copper signal (0.28–0.30 T). It is difficult to observe the hyperfine lines without the magnification of the Type II copper signal, as shown in Fig. 2. In the case of ascorbate oxidase containing Type I: Type II = 1:1, the Type II signal can be easily observed without the magnification of the signal [20], and hence the EPR spectrum of the nitrite reductase indicates that the amount of Type II copper is very low. However, the Type II copper is probably an intact form because of the A_z value (11.5 mT) similar to that (13.0 mT) of Type II copper in nitrite reductase from *Achromobacter cycloclastes* [21]. According to the recent X-ray crystal analysis of *Achromobacter* nitrite reductase, the enzyme contains one each of Type I and Type II coppers per one subunit, but the Type II copper site is located between two subunits [8]. Therefore, the Type II copper might be removed from the protein by the dissociation of the subunits [22]. In the case of *Hyphomicrobium* nitrite reductase, Type II copper would be also depleted partially during the preparation of the enzyme, but the enzyme activity still remains.

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