A copper-containing protein that inhibits nitrite reductase from *Pseudomonas aeruginosa*

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A non-blue copper-containing glycoprotein was isolated from *Pseudomonas aeruginosa*. The protein has a molecular mass of 10 kDa and contains 1 atom of EPR-detectable type II copper. The protein inhibits oxidation of both azurin and cytochrome *c*-551 catalyzed by nitrite reductase from *Ps. aeruginosa*. Thus, it may be considered as an endogenous inhibitor of nitrite reductase.

Copper-containing protein *(Pseudomonas aeruginosa)* *Enzyme inhibition* *Nitrite reductase*

1. INTRODUCTION

During searches for copper in different fractions of extracts obtained from *Pseudomonas aeruginosa* we observed a copper-containing protein fraction [1], the properties of which were significantly different from those of azurin, a blue copper-containing protein from *Ps. aeruginosa* [2,3]. Thus, *Ps. aeruginosa* was found to contain at least two copper-containing proteins: azurin and a novel protein in which copper belongs to the so-called 'non-blue' type. However the properties and the role of this novel copper protein were not studied.

Here we report about the purification and some physico-chemical properties of this protein. In particular, evidence is presented that the protein inhibits nitrite reductase (cytochrome cd_1, cytochrome oxidase) also obtained from *Ps. aeruginosa*. This finding seems to be the first indication of the presence in *Ps. aeruginosa* of a protein which is able to inhibit nitrite reductase.

2. MATERIALS AND METHODS

Nitrite reductase, azurin and cytochrome *c*-551 were obtained from anaerobically grown *Ps. aeruginosa* (IFO 3080) essentially according to Parr et al. [4]. All these preparations were electrophoretically homogeneous and had the following spectral ratios: $A^{ox}_{629}/A_{280} = 0.6$, $A^{red}_{551}/A_{280} = 1.14$ and $A^{ox}_{410}/A_{280} = 1.17$ for azurin, cytochrome and nitrite reductase, respectively. The oxidation of azurin and cytochrome *c*-551 by nitrite reductase was followed by an increase of intensity of the band at 629 nm characteristic for oxidized azurin and a decrease of the band at 551 nm characteristic for reduced cytochrome. Both reactions were carried out at 20°C in 0.01 M phosphate buffer containing 10^{-4} M EDTA. Kinetic curves and optical spectra were obtained on a Specord M-40 spectrophotometer (GDR) using 0.2 cm cells.

EPR spectra were taken at 77 K on a Varian E-4 instrument operating at microwave frequency, 9.08 GHz; microwave power, 10 mW and modulation amplitude, 10 G.

The copper content was assayed according to Matsuba and Takahashi [5] for three separate preparations. Protein was determined by the method of Lowry et al. [6]. Carbohydrate content in protein preparations was evaluated according to Dische [7] and Stumpf [8]. Molecular mass of the protein was determined electrophoretically [9].

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3. RESULTS AND DISCUSSION

3.1. *Preparation of the protein*

Routinely, 250 g of wet paste of *Ps. aeruginosa* was suspended in 1.2 l of 0.02 M phosphate buffer, pH 7.4 and 300 ml portions were sonicated at 22 kHz for 10 min. During sonication the temperature of the suspension was maintained 5–6°C. Then the suspension was centrifuged at 10000 × *g* for 30 min and to the supernatant obtained solid ammonium sulphate was added to 45% saturation. The residue formed was removed by centrifugation, and to the supernatant ammonium sulphate was added to full saturation. The residue formed was suspended in 150 ml of 0.001 M phosphate buffer, pH 7.4, and the suspension was further dialyzed against 40 l distilled water during 24 h and, after clarification by centrifugation, it was passed through a column (5.5 × 7) with DEAE-cellulose equilibrated against 0.005 M Tris-HCl buffer, pH 7.8. The charged column was washed with the same buffer, then with 0.05 M buffer, and a yellowish copper-containing fraction was sharply eluted with 0.1 M Tris-HCl buffer, pH 7.8. This fraction was dialyzed, concentrated and then gel filtered through a column with Sephadex G-75 (superfine). Copper-containing fractions were collected, concentrated by ultrafiltration and finally purified by gel filtration through Sephadex G-50 (superfine). At this stage the elution pattern represents the only symmetric peak in which the maximum at 260 nm coincides with the maximal content of copper. This preparation was found to be electrophoretically homogeneous when analyzed in 10% polyacrylamide gel (fig.1). The procedure used results in 10–12 mg of homogeneous preparation from 250 g of wet paste of *Ps. aeruginosa*.

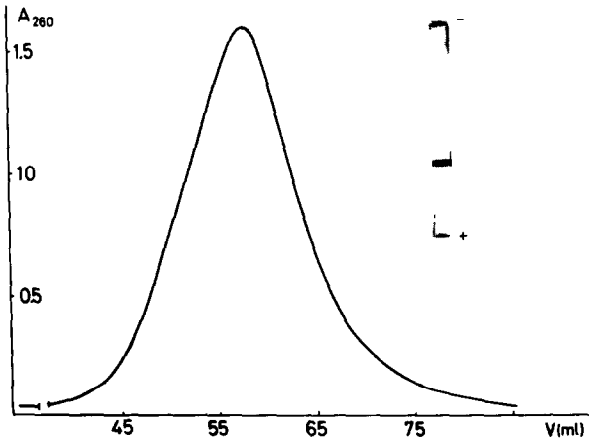

Fig.1. Electrophoretogram of the purified protein and the elution pattern of the preparation during gel filtration through Sephadex G-50 at the last step of the purification. The column (1.5 × 60) was equilibrated with 0.1 M Tris-HCl buffer, pH 7.8. Fractions were collected after elution of the external volume of the column.

3.2. *Properties of the protein*

The main maximum in the UV spectrum of the purified protein is located at 260 nm. There is also a minor absorbance at 330 nm (see fig.2). At the same time no absorbance in the visible region, and in particular at 630 nm characteristic for azurin, was observed. The molecular mass of the protein was found to be 10 kDa. It follows from this value

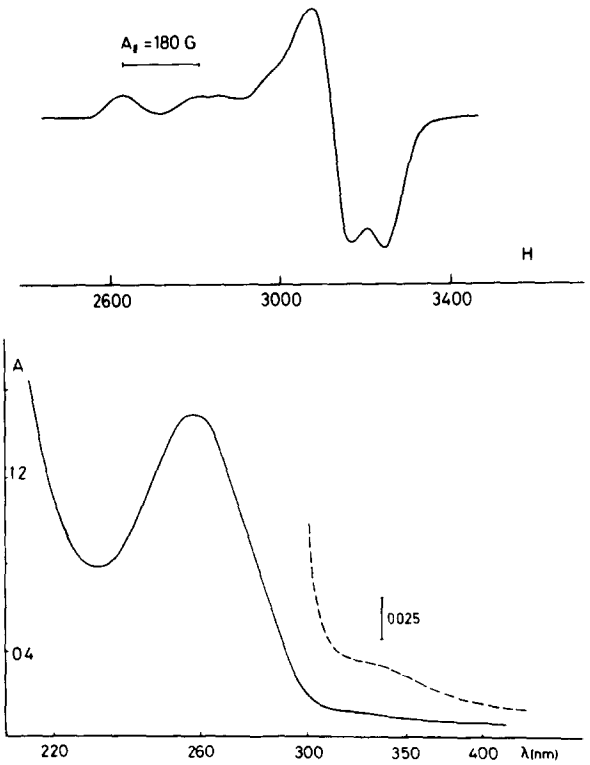

Fig.2. EPR (above) and optical (below) spectra of the copper protein. The EPR spectrum was recorded at pH 7.4. Parameters of the signal were: $g_m = 2.07$; $g = 2.23$; $A = 180$ G. The part of the optical spectrum at 300–400 nm recorded at the higher sensitivity is shown by a dashed line.

and from assays of the copper content in the preparation that the protein contains 1.1–1.3 copper atoms per molecule. The copper in the protein is EPR-detectable (fig.2). The shape of the EPR signal depends on pH. Parameters of the EPR signal are characteristic of a so-called type 2 copper (non-blue copper). It is of importance to stress once more that the EPR spectrum of azurin is typical of a type 1 copper (blue copper) [10]. The reduced form of the protein was practically non-auto-oxidizable.

The protein gave a positive reaction when its total carbohydrate content was tested; the fructose test was also positive. Thus, the preparation isolated is a copper-containing glycoprotein.

It is well known that nitrite reductases of many denitrifying microorganisms, and among them *Ps. aeruginosa*, have also cytochrome *c*- and azurin-oxidase activities [11–13], i.e. oxygen, instead of nitrite, may be used as a terminal acceptor.

Here we found that the oxidation of azurin as well as cytochrome *c*-551 catalyzed by nitrite reductase is inhibited by the protein isolated from *Ps. aeruginosa*. These results are shown in fig.3, where the Dixon plot obtained for the oxidation of cytochrome *c*-551 is also presented. The inhibition constant was determined to be 2.5×10^{-6} M indicating a potent inhibition of the enzyme by the protein. The effect of the protein was completely reversible because its removal from the reaction mixture by gel filtration resulted in the initial activity of nitrite reductase. The inhibition seems not to be connected with the interaction of the protein with substrates of the enzyme. In particular, in the absence of the enzyme under both aerobic and anaerobic conditions we were unable to observe any redox reactions between the protein and azurin or cytochrome *c*-551.

A final conclusion on the mechanism of the inhibition of nitrite reductase by the protein as well as its physiological function remains to be settled. There are also many problems concerning the structural features of this novel protein. However it may be suggested at present that the protein seems to be an endogenous inhibitor of nitrite reductase in *Ps. aeruginosa*. It is of interest,

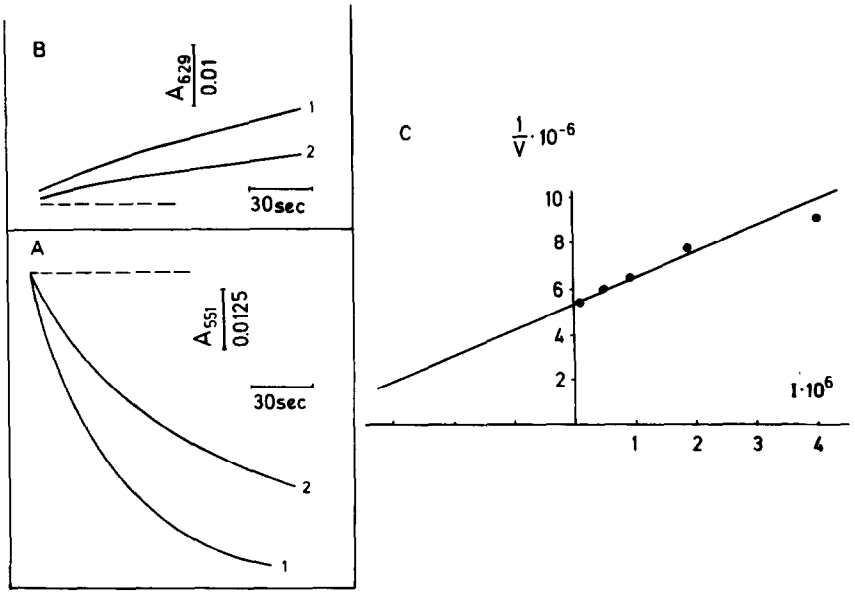

Fig.3. The effect of the copper protein on the oxidation of cytochrome *c*-551 (A) and azurin (B) by nitrite reductase and the Dixon plot obtained for the oxidation of cytochrome *c*-551 (C). The concentration of nitrite reductase in (A) and (B) was 1.5×10^{-7} M. For curves 1 and 2 concentrations of cytochrome *c*-551 and azurin were 1.6×10^{-5} M and 2.35×10^{-4} M, respectively. Curves 2 were recorded when 4.4×10^{-7} M of the copper protein was added to mixtures of nitrite reductase with substrates. Dashed lines (controls) represent kinetics obtained in the absence of nitrite reductase. In (C) concentrations of cytochrome *c*-551 and nitrite reductase were 3.2×10^{-5} and 1.5×10^{-7} M, respectively.

therefore, to determine whether other denitrifiers contain a similar protein inhibitor.

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