A CYTOCHROME cc'-LIKE HAEMOPROTEIN ISOLATED FROM *Azotobacter vinelandii*

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Summary: From *Azotobacter vinelandii*, a cytochrome cc'-like haemoprotein has been obtained. It shows an absorption peak at 397 nm in the oxidized form. On reduction with $Na_2S_2O_4$, a peak appears at 423 nm and a broad band around 550 nm. The reduced form combines with CO, whereas the oxidized form combines with NO. In an alkaline solution, the absorption spectrum of the haemoprotein changes into the haemochrome-type. Although the haemoprotein is autoxidizable, it does not show cytochrome oxidase activity with cytochrome c_4 or c_5 as the electron donor. No biological function of the haemoprotein has been found so far.

Cytochrome cc' (or c') has been isolated from the photosynthetic bacteria such as *Rhodospirillum rubrum* (1), *Chromatium* D (2) and *Rhodopseudomonas palustris* (3, 4), and from the denitrifying bacterium, *Pseudomonas denitrificans* (5). However, it has not been obtained from any other bacterium so far. Recently, we have succeeded in isolation from *Azotobacter vinelandii* of a haemoprotein with many properties similar to those of cytochrome cc'. This fact may provide a clue to elucidate the function of cytochrome cc' or c'.

Cells of *A. vinelandii* IFO 12018 cultivated in the nitrogen-free Burk's medium (6) were suspended in 10 mM Tris-HCl buffer, pH 8.5 and treated for 10 min by sonication (20kc, 400 W). The sonicate obtained was 10%-saturated with $(NH_4)_2SO_4$, allowed to stand for an hour at 4°, and centrifuged at 10,000 x g for 30 min. The resulting supernatant was fractionated by $(NH_4)_2SO_4$, the

precipitate appeared between 40% and 90% saturation collected by centrifugation and dissolved in 10 mM Tris-HCl buffer, pH 8.5. The solution thus obtained was subjected to chromatography with a DEAE-cellulose column after dialysis against the same buffer as used to dissolve the precipitate. The haemoprotein appeared as a dark brown band after adsorption on the column together with other coloured materials. After the cellulose column had been washed with 10 mM Tris-HCl buffer, pH 8.5, containing 50 mM NaCl, the haemoprotein was eluted with 10 mM Tris-HCl, pH 8.5, containing 0.1 M NaCl. The eluate thus obtained was further subjected to the chromatography with the DEAE-cellulose column which had been equilibrated with 10 mM Tris-HCl, pH 8.5, containing 50 mM NaCl, and the haemoprotein adsorbed on the column was eluted with 10 mM Tris-HCl buffer, pH 8.5, containing 0.1 M NaCl. The resulting eluate was fractionated with $(NH_4)_2SO_4$, and the precipitate formed between 60% and 70% saturation was collected by centrifugation. The pre-

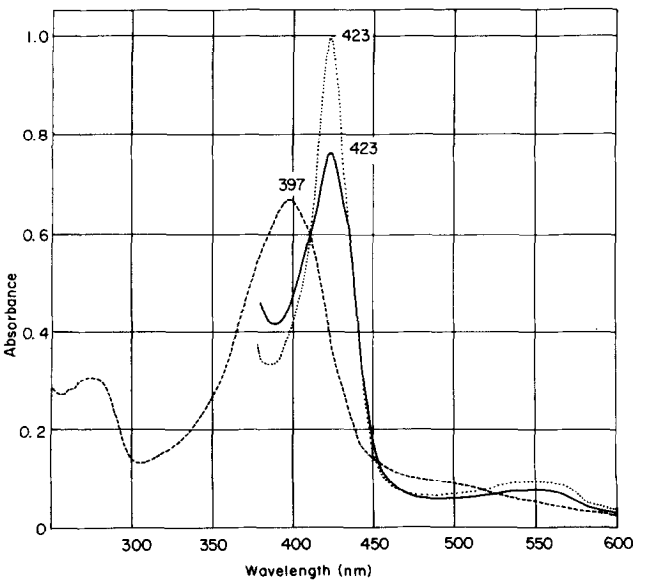

Fig. 1 Absorption spectra of A. vinelandii cytochrome cc'-like haemoprotein. -----, Oxidized; ———, reduced with $Na_2S_2O_4$; ·····, reduced form was bubbled with CO.

ciptate thus obtained was dissolved in an appropriate buffer and the resulting solution was subjected to further purification by chromatography with Sephadex G-75 and by isoelectric fractionation (7). The purified preparation of the haemoprotein thus obtained was used for the determination of various properties.

As Fig. 1 shows, the oxidized form of the haemoprotein possessed a broad γ band at 397 nm with a shoulder around 500 nm and a small broad band around 630 nm although the latter is not shown in the figure. On reduction with $Na_2S_2O_4$, the γ band appeared at 423 nm with a shoulder at 433 nm, and a broad band around 550 nm. The reduced form combined with CO resulting in increase in the extinction of the γ band at 423 nm (Fig. 1), while the oxidized form combined with NO. The NO-complex showed absorption peaks at 562, 532 and 418 nm. As cytochrome cc' reacts with NO in both oxidized and reduced forms (8), A. vinelandii haemoprotein obtained here differs from the cytochrome in this respect.

In an alkaline solution (2N NaOH), the spectrum of the reduced form changed into the haemochrome-type with peaks at 551, 523 and 418 nm (Fig. 2). Although this change occurred even at the NaOH concentrations less than 2 N, such formation of the haemochrome-type spectrum was not complete. Thus, on neutralization of the alkaline solution, the haemoprotein regained its original spectrum, while the spectrum of the protein treated with 2 N NaOH retained the haemochrome-type even after the solution was neutralized. The pyridine haemochrome was not fully formed in the routine way, i.e. by addition of 0.1 N NaOH and 10% pyridine. Addition of 0.05% SDS to the haemoprotein solution dissolved in 0.4 N NaOH plus 10% pyridine was required to complete the formation of the pyridine haemochrome. After reduction with $Na_2S_2O_4$, the absorption spectrum of the resulting ferrohaemochrome showed that the haemoprotein pos-

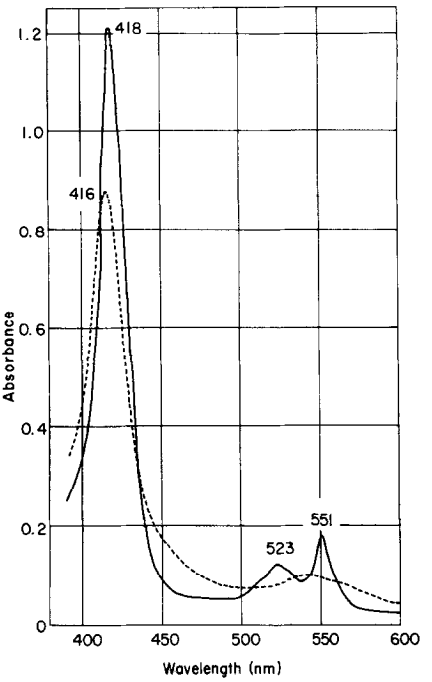

Fig. 2 Effect of 2 N NaOH on absorption spectrum of A. vinelandii cytochrome cc'-like haemoprotein. -----, Oxidized; ———, reduced with $Na_2S_2O_4$.

sessed haem c; the absorption peaks were at 551, 523 and 415 nm. The absorption spectrum of the reduced haemoprotein was also affected by 50% propyl alcohol and by 0.02% SDS; slight peaks appeared at 551 and 523 nm. However, the protein seemed to be less soluble in alcohol than the other cytochromes cc' (9). Thus, in 50% propyl alcohol, the protein precipitated after several min standing.

In isoelectric fractionation (7), the haemoprotein preparation formed a single band at pH 4.7. Therefore, the preparation seemed to be electrophoretically homogeneous. By gelfiltration with Sephadex G-150 column (10), the molecular weight of the haemoprotein was determined to be 170,000.

Although the haemoprotein was markedly autoxidizable, it did not catalyse oxidation of reduced form of cytochrome c_4 or c_5.

Neither (guaiacol) peroxidase activity nor cytochrome peroxidase activity was found associated with the haemoprotein. In determination of the latter activity, the reduced form of cytochrome c_4 or c_5 was used as the electron donor. No biological function of the haemoprotein has been found so far.

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