Purification and properties of cytochromes c_4 and c_5 from *Azotobacter vinelandii*

The purpose of this communication is to describe the purification and properties of two cytochromes from *Azotobacter vinelandii*. These two components resemble cytochrome *c* in several important properties and for this reason they are called here cytochromes c_4 and c_5. They were extracted from the cells by means of *n*-butanol[1].

One part of *Azotobacter vinelandii* cells was mixed in a Waring blendor for 1–2 minutes with one part of *n*-butanol (w/v); the butanol was decanted off and 1.2 parts of water were added to the cells, the mixture was blended again and centrifuged for 30 minutes at 10,000 r.p.m. The clear pink layer containing the cytochromes was pipetted off and the residue was extracted once more with the same amount of water. The pink solution from both extractions was dialysed to remove the butanol and centrifuged. The pH of the clear solution was brought to pH 8 with 0.1 *N* NaOH and a solution of 25% basic lead acetate was added till no further precipitate was formed. The precipitate was centrifuged off and discarded and the excess of lead was removed by the addition of sodium sulphate at pH 6.5, or by dialysis. Two volumes of neutralized saturated ammonium sulphate were then added at pH 7. A reddish precipitate was formed which was collected by centrifugation, dissolved in a small volume of 0.02 *M* phosphate buffer pH 7.5, dialysed first against the same buffer, then against distilled water, and freeze dried. Paper electrophoresis was performed on Whatman No. 3 mm paper, with 0.05 *M* tris buffer pH 8 and a current of 200 volts. The pink dried material was dissolved in the minimum amount of buffer and applied as a narrow band across the strip of wet paper, 8 inches wide and 15 inches long. The paper, between the two large electrode vessels into which it dipped, was laid across a shallow pyrex dish, covered with a glass plate. The paper was so arranged that it hung clear of the glass plate. The pigment moved towards the positive electrode and separated into two coloured bands: the faster moving one, cytochrome c_4, comprising about 3/4 of the total pigment, had moved about 10 cm in 15 hours; the second one, cytochrome c_5, lagged behind by 4–5 cm. They were eluted separately. The cytochrome c_5 fraction often contained very small amounts of cytochrome c_4 which were separated by a second paper electrophoresis. While the position of their joint α-absorption band lies at about 552 mμ in the crude extract or in the intact cells, that of cytochrome c_4 is situated at 551 and that of cytochrome c_5 at 555 mμ.

The absorption spectrum of purified cytochrome c_4 closely resembles that of pure cytochrome *c* (*cf.* ref.[2]). The absorption bands of the reduced compound have the following positions: α: 551 mμ; β: 522 mμ; γ: 416 mμ; δ: 315 mμ. The γ-band of ferricytochrome c_4 lies at 411 mμ. The m*M* extinction coefficients of the various absorption bands, calculated on the basis of the iron content (see below) are: α: 23.8; β: 17.6; γ (reduced): 157.2; γ (oxidized): 115.8; protein band (270 mμ): 20.5. Thus the extinction coefficient of the α-band is lower, while that of the γ-band is higher than the corresponding values for cytochrome *c*. The extinction coefficient of the protein band is lower than that of cytochrome *c* containing 0.45% iron (*cf.* ref.[2]).

The same sample of cytochrome c_4 was dialysed for 48 hours against glass-distilled water and freeze dried. Iron was estimated by the $\alpha\alpha'$-dipyridyl method[3], and nitrogen by Kjeldahl. The iron content was found to be 0.46% of the dry weight or of the amount of protein. Therefore the molecular weight, calculated on the basis of one atom of iron per molecule, is about 12,000. These values are very similar to those obtained for cytochrome *c*[4].

The spectrum of cytochrome c_6 is very similar to that of c_4, the whole spectrum being shifted towards the longer wavelengths by 4 mμ.

The following properties apply to both cytochrome c_4 and c_5. The pigments in solution remain partly reduced after extraction from the cells. They are not autoxidizable nor do they combined with carbon monoxide between pH 4.5 and 13. They are not affected by heating for 10 minutes in a boiling water bath. They are remarkably stable in alkali, no change being observed after the addition of 1 volume of *N* NaOH; when pyridine was added to this alkaline solution, the α-band of either c_4 or c_5 shifted to 550 mμ. The pigments are denatured when kept below pH 4.5 for several hours. Unlike cytochrome *c*, the bacterial cytochromes do not adsorb on Amberlite IRC-50.

Between pH 8 and 5.5 the cytochromes moved towards the positive electrode on paper electrophoresis, which shows that both c_4 and c_5 have an isoelectric point in the acid range. Cytochrome c_5 lagged behind cytochrome c_4 which suggests that its isoelectric point is somewhat higher than that of c_4.

The oxidation-reduction potential was determined using mixtures of ferro- and ferricyanide as oxidation-reduction buffers according to DAVENPORT AND HILL[5]. It was found that the potential of half reduction, E_0', for cytochrome c_4 was + 0.30 volts between pH 5.5 and 7.3, while for cytochrome c_5 it was + 0.32 volts between pH 5.8 and 7.3.

The activities of cytochromes c_4 and c_5 were studied in the succinic oxidase and cytochrome oxidase systems of *Azotobacter vinelandii* preparations. The extract was made as described previ-

ously[6], and a particulate fraction, not sedimented by 30 minutes centrifugation at 15,000 *g* but sedimented during 60 minutes at about 120,000 *g* was obtained. It was washed in 0.05 *M* phosphate buffer pH 7, centrifuged again and resuspended in the same buffer. This preparation contains all the cytochrome pigments of the original cells. They are all present in the oxidized form and their absorption bands are thus not observable spectroscopically except that of oxidized cytochrome a_2 at 645 mμ. On addition of a suitable reducer a strong ($c_4 + c_5$) band and a weak b_1 band appear at 552–552.5 mμ and 560 mμ respectively and the band of cytochrome a_2 moves to 630 mμ. All the components of cytochrome can be reduced either by succinate, or by reduced diphosphopyridine nucleotide (DPNH), and oxidized on shaking in air. No other substrate was found to be effective. When reduced purified cytochrome ($c_4 + c_5$), c_4 or c_5 are added in the absence of substrate the exogenous pigments become oxidized at once. If then succinate or DPNH is added the exogenous pigments become reduced and can undergo oxidation on shaking in air and reduction on standing.

Succinic and cytochrome oxidase activities were measured in micro differential manometers at 30° in the presence of air and KOH. The manometric flask contained the enzyme in 0.05 *M* phosphate buffer pH 7, and eithers uccinate or, in the case of cytochrome oxidase, ascorbate was tipped in from the side arm to give a final concentration of 0.02 *M*. The total volume of the reaction mixture was 0.5 ml. Under those conditions, the Q_{O_2} with succinate was about 1000. It was not modified by the addition of cytochrome c_4, c_5 or *c*. With ascorbate, the system did not take up any oxygen unless *Azotobacter* cytochrome was added. The oxygen uptake was proportional, within certain limits, to the amount of exogenous cytochrome c_4 or c_5 present, and with $4 \cdot 10^{-5}$ *M* cytochrome c_4, the Q_{O_2} was 500–600. Cytochrome c_5 had approximately the same effect. Cytochrome *c*, *Rhodospirillum rubrum* cytochrome *c* or cytochrome *f* were ineffective. Cytochrome c_4, and a mixture of cytochrome c_4 and c_5, were tested manometrically and spectroscopically in the succinic and cytochrome oxidase systems of cytochrome *c*-deficient heart muscle preparations[7]. They had no effect on either of those two systems, while the addition of cytochrome *c* produced, as expected, a large oxygen uptake. KAMEN AND VERNON[8] also reported that the *c* type cytochrome and its oxidase from *Azotobacter vinelandii* reacted only with each other and could not be linked to the cytochrome *c* – cytochrome oxidase system from mammalian cells. Cytochrome c_4 was also tested with particulate preparations from *Acetobacter peroxidans* made as described above for *Azotobacter* and it was found to have no activity.

It is known that a mixture of two cytochrome components, the α-bands of which differ in position by only 4 mμ, may show a symmetrical α-band lying at an intermediate position[9] and it is therefore not surprising that the presence of the two cytochromes cannot be detected in *Azotobacter* cells or in crude extracts. Cytochrome c_5 differs from cytochrome c_4 in three respects: it has a somewhat higher isoelectric point and oxidation reduction potential, and the absorption bands lie 4 mμ nearer the red end of the spectrum. It is possible that c_5 plays in *Azotobacter* a role similar to that of c_1 in animal tissues[9] and it is conceivable that the respiratory chain uses c_5 for the oxidation of some specific substrates. The similarity of cytochrome c_4 and c_5 with cytochrome *c* is striking. However, the differences in the isoelectric point or in the oxidation-reduction potential between cytochrome *c* on one hand and cytochrome c_4 and c_5 on the other may be sufficient to account for their specific biological properties.

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