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The Oxidation-Reduction Potentials of Cytochrome $o + c_4$ and Cytochrome o Purified from *Azotobacter vinelandii*

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Oxidation–reduction titrations of *Azotobacter vinelandii* cytochrome $o + c_4$ and cytochrome o were performed with simultaneous potential and absorbance measurements under anaerobic conditions. Cytochrome c_4 has a midpoint potential ($E_{m,7.4}$) of 260mV and purified cytochrome o has an $E_{m,7.4}$ of −18mV. Little change in the midpoint potential of cytochrome o was observed when titrated in the pH range 6.2–9.8.

Cytochrome *o*, a distinct haemoprotein that binds CO and acts as an oxidase in some bacteria, was first recognized and reported in this laboratory some 20 years ago (Chance *et al.*, 1953; Castor & Chance, 1959). However, little has been known about its chemical and physical properties besides its physiological function being an oxidase. Until recently, there were only two cytochrome *o* components that had been reported to be isolated from prokaryotic organisms. An apparent soluble cytochrome *o* has been purified from a gliding bacterium, *Vitreoscilla*, by Webster and his co-workers (Webster & Hackett, 1966; Tyree & Webster, 1978*a*). This cytochrome *o* has a molecular weight of about 27000. It contains two polypeptide chains of equal molecular weight and consists of two haem groups, with different oxidation–reduction potentials (midpoints at 118mV and −122mV at pH7) (Tyree & Webster, 1978*b*). Another cytochrome *o*, a membrane-bound type, has been isolated and purified from *Azotobacter vinelandii* and demonstrated to be a typical *b*-type cytochrome in both spectral and haem properties (Yang & Jurtshuk, 1978*a*). Preliminary results indicate that this solubilized cytochrome *o* consists of only one polypeptide with a molecular weight about 28000 and contains two identical haem components (T. Yang, unpublished work). In the present communication we report on the oxidation–reduction titration of this cytochrome component both in a partially purified preparation, which also contains cytochrome c_4, and in its purified form.

Materials and Methods

Cytochrome *o*, in partially pure (cytochrome $o+c_4$) and pure forms, was prepared essentially according to the methods of Yang & Jurtshuk (1978*b*). The preparations were then passed through a column (1.5cm×36cm) of Sephadex G-50 pre-equilibrated with 0.02M-potassium phosphate buffer, pH7.4, containing 0.05% Triton X-100. Fractions were pooled and then concentrated by vacuum dialysis before the titration.

Oxidation–reduction titrations were performed in a vessel described by Dutton (1971). Spectra were recorded on a scanning dual-wavelength spectrophotometer interfaced with a Digital PDP 11/10 computer for standard difference spectroscopy. The redox mediators used in the titration experiments were diaminodurol, phenazine methosulphate, phenazine ethosulphate, pyocyanine and 2-hydroxy-1,4-naphthaquinone. Both $K_3Fe(CN)_6$ and $Na_2S_2O_4$, which were added to the cytochrome preparations to obtain either more positive or more negative potentials, were prepared freshly for each experiment. The buffer used to dilute the cytochrome preparations was de-aerated by bubbling with argon for 20min, and during titration the anaerobic cuvette was continuously flushed with argon gas. When the redox titration was performed in the presence of CO the preparations were first reduced completely and then kept under anaerobic conditions before and during bubbling with CO.

The purified cytochrome *o* preparation was assayed for purity by analytical polyacrylamide-gel electrophoresis by the method of Davis (1964), with a 4% gel containing 0.05% Triton X-100.

Protein concentrations were determined either by the method of Lowry *et al.* (1951) or by a modified method with inclusion of 5% (w/v) sodium dodecyl sulphate in the preparations (Wang & Smith, 1975). The standard used was bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.).

Results and Discussion

It was previously reported that a *c*-type cytochrome was co-purified with cytochrome *o* from the

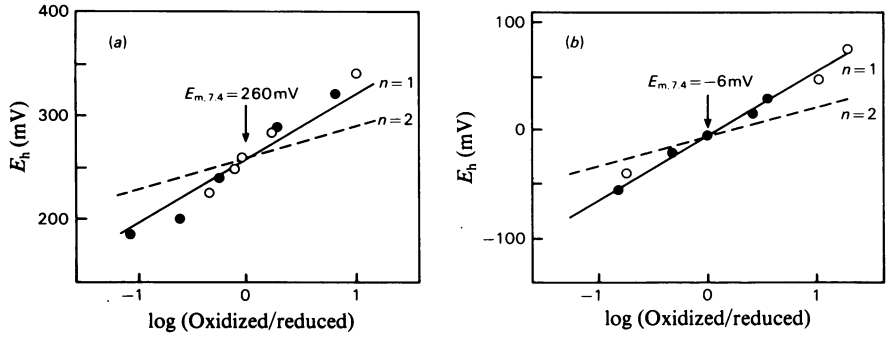

Fig. 1. *Redox titration of (a) the component in the partially purified cytochrome o preparation absorbing maximally at 553 nm (cytochrome c_4) and (b) the component in the partially purified cytochrome o preparation absorbing maximally at 558 nm (cytochrome o)*

The sample contained 2mg of protein/ml in 25mM-potassium phosphate buffer, pH 7.4, containing 20μM-phenazine methosulphate, 20μM-phenazine ethosulphate, 20μM-diaminodurol, 5μM-pyocyanine and 5μM-2-hydroxy-1,4-naphthaquinone. The titration was performed as described in the Materials and Methods section. ●, Reductive titration; ○, oxidative tritration.

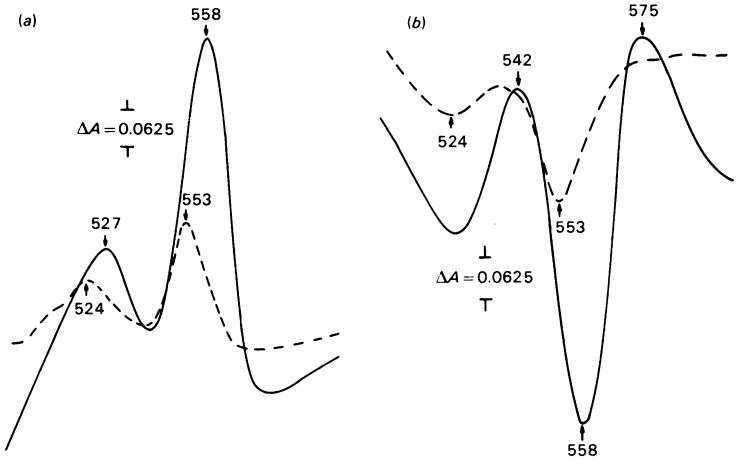

Fig. 2. (*a*) *Reduced-minus-oxidized difference spectra of cytochromes o and c_4 in the cytochrome o+c_4 preparation and (b) CO difference spectra of the cytochrome o+c_4 preparation*

(*a*) Conditions were the same as for Fig. 1, and the redox potentials used to resolve the two spectra were determined from the redox titration in Fig. 1. ——, −368mV minus +115mV (cytochrome *o*); ----, +115mV minus +400mV (cytochrome c_4). (*b*) The sample was prepared as in (*a*) and reduced with dithionite, the spectra were recorded and then the sample was bubbled with CO for 3min. ——, Reduced CO-treated (−317mV) minus reduced (−370mV) cytochrome sample; ----, oxidized CO-treated (+307mV) minus reduced CO-treated (−317mV) cytochrome sample.

membrane of *A. vinelandii* (Yang & Jurtshuk, 1978*a*). The presence of this *c*-type cytochrome is of interest for several reasons. First, it has been proposed that cytochrome *o*, in association with a *c*-type cytochrome (c_4 and/or c_5), functions as a branched cytochrome oxidase complex in the *A. vinelandii* electron-transport chain (Jones & Redfearn, 1967). This branch of the chain has a distinguishing characteristic of high sensitivity towards cyanide and light. Secondly, the cytochrome *c+o* chain has been shown to be very active in oxidizing reduced *NNN'N'*-tetramethyl-*p*-phenylenediamine and 2,6-dichlorophenol-indophenol (Jurtshuk *et al.*, 1978). Thirdly, recent evidence indicates that cytochrome *c+o* has been isolated as an *NNN'N'*-tetramethyl-*p*-phenylenediamine oxidase complex (Jurtshuk *et al.*, 1978). During the present study it became evident that these two components (cytochromes *o* and *c*), although co-solubilized from the membrane of *A. vinelandii*, exhibited very different redox potentials.

This was apparent when the addition of ascorbate reduced cytochrome *c* readily, yet left cytochrome *o* in the oxidized form, an indication of a relatively high-potential *c* component and a low-potential *o* component. Kauffman & van Gelder (1973) have also suggested this to be the case, on the basis of kinetic studies.

A redox titration performed on the partially purified cytochrome *o* preparation indicated that only one *b*-type and one *c*-type cytochrome component could be observed in the potential range from −300 to +400mV. The titrations of each cytochrome are shown in Figs. 1(*a*) and 1(*b*). When titrated in the partially purified cytochrome *o* preparation the *c*-type cytochrome has a midpoint potential of +260mV (see Fig. 1*a*). Although the reduced α-absorption peak of this *c*-type cytochrome resembles a combination of the spectra of cytochromes c_4 and c_5 (Swank & Burris, 1969), the cold-temperature (liquid N_2) spectrum (results not shown) and redox titration (shown in Fig. 1*a*) suggest the presence of only one component. The cold-temperature spectrum is more characteristic of cytochrome c_4, and consequently we have tentatively identified this *c*-type cytochrome as cytochrome c_4. The midpoint we have determined is somewhat lower than that reported for purified cytochrome c_4 isolated by a different technique (Tissières, 1956). Fig. 1(*b*) shows that the midpoint potential of cytochrome *o*, titrated in the presence of cytochrome c_4, is −6mV.

With knowledge of the midpoints of cytochromes *o* and c_4, it is possible to separate spectrally the two components in a mixture by poising the E_h such that reduced-minus-oxidized difference spectra may be taken of each component individually. Fig. 2(*a*) shows the spectra of cytochromes *o* and c_4 obtained in this manner. The amount of cytochrome c_4 is estimated to be about 25–30% of the total cytochrome in the preparation, and is identical with the amount of *c*-type cytochrome that is reducible by ascorbate only. To determine that cytochrome c_4 has no reaction with CO, and hence that in a preparation such as cytochrome $o+c_4$ the cytochrome *o* is the sole CO-reacting component, an oxidized-minus-reduced difference spectrum was taken for the CO-saturated preparation. The CO difference spectrum, shown in Fig. 2(*b*), with absorption peaks at 542 and 575nm and a prominent trough at 558nm in the visible region, is characteristic of a cytochrome *o* spectrum (Chance *et al.*, 1953; Yang & Jurtshuk, 1978*a*). When the potential of the CO-saturated (reduced) cytochrome preparation is raised to a value where both unliganded cytochromes *o* and c_4 should be oxidized, only the oxidized form of cytochrome c_4 is observed. The amount of cytochrome c_4 oxidized in this experiment corresponds to the total amount of cytochrome c_4 present (compare Figs. 2*a* and 2*b*). It is therefore concluded that the low-potential component of the complex is cytochrome *o*, and it is the only component that reacts with CO. This confirms the previously reported result that cytochrome c_4 in its purified form has no reactivity with CO (Swank & Burris, 1969). The possible presence of other CO-binding components such as a cytochrome *cc'* (Yamanaka & Imai, 1972) or a CO-reacting cytochrome c_5 (Swank & Burris, 1969), which have been isolated from the membrane of *A. vinelandii*, is also eliminated.

A redox titration of purified cytochrome *o* is shown in Fig. 3. The midpoint of the purified preparation is found to be −10mV. This value is quite close to the −6mV midpoint determined in the partially purified preparation, and suggests that the final purification steps have little or no effect on the midpoint of the cytochrome.

The effect of pH on the midpoint of cytochrome *o* is shown in Fig. 4. In the pH range 6.2–9.8 the

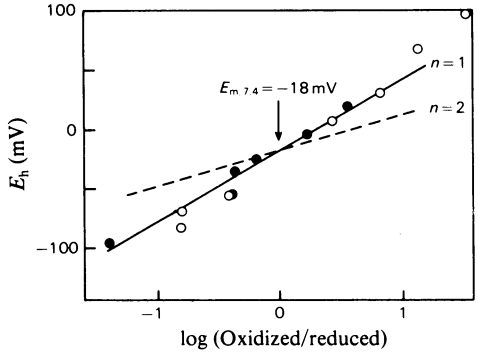

Fig. 3. *Redox titration of purified cytochrome o*
Conditions were the same as for Fig. 1 except that the final concentration of cytochrome *o* was 2.8μM.

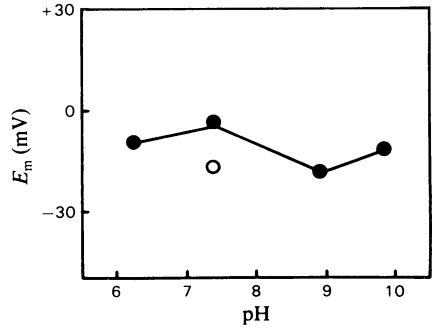

Fig. 4. *pH-dependence of the midpoint of cytochrome o*
Conditions were the same as for Fig. 1(*a*) except that the buffers used were 50mM-4-morpholineethanesulphonic acid (below pH6.5) and 50mM-glycine (above pH8.8). ●, Partially purified cytochrome *o*; ○, purified cytochrome *o*.

value of the midpoint varied little, and certainly did not exhibit a 60mV/pH unit variation as would be expected if a proton were involved in the transfer of an electron to and from cytochrome *o*. The pH-independence of the midpoint indicated that in this pH range the redox reactions of cytochrome *o* did not involve a proton-binding reaction.

There is some evidence that cytochrome c_4 in the membrane has a high midpoint potential (Kauffman & van Gelder, 1973). From the present data it seems that solubilization and purification have no major effect on the midpoint of cytochrome c_4. Since both cytochromes are tightly membrane-bound, we assume that in its isolated form cytochrome *o* also has a midpoint close to that of the membrane-bound form. If this is the case, it is difficult to assess a direct functional connection between these two cytochromes (c_4 and *o*), considering the nearly 300mV potential gap between them. This is especially difficult in electron-transfer schemes where cytochrome c_4 at +260mV is to reduce cytochrome *o* at −18mV. However, it seems clear that cytochrome *o* preparations with enhanced amounts of cytochrome c_4 have an increased *NNN'N'*-tetramethyl-*p*-phenylenediamine oxidase activity (Jurtshuk *et al.*, 1978), which would imply that cytochrome c_4 functions as the physiological reductant of cytochrome *o*, although thermodynamically this seems unlikely.

The present results do seem to support the role of cytochrome *o* as a terminal oxidase. However, its midpoint is so low that two very important questions are raised. (1) What would be the advantage of such a large free-energy release on reduction of O_2 by cytochrome *o*? (2) If cytochrome *o* functions in a portion of a normal linear electron-transport chain, then what is its physiological reductant?

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