MID-POTENTIAL MEASUREMENTS OF AN UNIDENTIFIED COMPONENT, CONTROLLING THE RATE OF REDUCTION OF THE *b*-TYPE CYTOCHROMES

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1. Introduction

The biphasic reduction of the *b*-type cytochromes in the respiratory system was investiged by this laboratory. It was proposed that the biphasic reduction was a consequence of a dynamic control mechanism, which transformed the *b*-type cytochromes from their kineticly active form to a sluggish one [1]. This transformation was attributed to a hypothetical control factor, called *Y*. When *Y* was oxidized, the *b*-type cytochromes were kineticly active; reductions of *Y* transformed them to their sluggish form.

In this paper we utilized the rate of the reduction of the *b*-type cytochromes to measure the ratio of $b_{active}/b_{sluggish}$, a value which we related to Y_{Ox}/Y_{Red}. Assuming that cytochrome *c* was in equilibrium with *Y*, the correlation between the redox levels of these two components enabled us to evaluate the mid-potential of *Y*.

2. Materials and methods

All the materials, techniques and procedures were as previously described [1].

3. Results and discussion

In order to measure the E_m of *Y* it was necessary to correlate its redox state with that of a mediator, either external or internal, which was in redox-equilibrium with *Y*. Since we could not find an external mediator which equilibrated with *Y* [2–4], we utilized the electron-transport carriers both as mediators and as redox indicators, in a mode similar to that which was used by Storey [5]. It was previously shown, that *Y* could be reduced by cytochrome *c* in an 2-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) – sensitive reaction, which could be reversed by addition of $K_3Fe(CN)_6$ [6]. Thus, it was assumed that *Y* and cytochrome *c* equilibrate with each other. There is no direct method for determination of the redox state of *Y*, but it can be evaluated from kinetic analysis of the reduction of the *b*-type cytochromes. Therefore a system was devised, which enabled us to measure the kinetics of the reduction of cytochrome *b*, while the redox state of the *c*-type cytochromes was predetermined and invariable during the reaction. This could be achieved by introduction of antimycin which divided the electron-transport chain into two sections. The section on the oxygen side of the antimycin block could be kept at a reduced steady-state with ascorbate plus TMPD (aerobically). Under these conditions the *b*-type cytochromes were mostly oxidized, since their reduction by TMPD was slow, and below 150 μM TMPD they were not reduced at all. Addition of succinate under these conditions caused a reduction of all the carriers on the substrate side of the antimycin block, and the rate of the reduction of the *b*-type cytochromes was measured. Furthermore, the uninhibited electron flux from cytochrome *c* to oxygen ensured that succinate could not affect the redox state of the *c*-type cytochromes. Consequently the monitoring system did not perturb the regulating system.

We assumed that in the respiratory chains in which *Y* was oxidized, cytochrome *b* was in its active form

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and was rapidly reduced. In those in which Y was reduced, cytochrome b was in its sluggish form and its reduction was slow [1]. Thus, quantitative analysis of the kinetics of the reduction of the b-type cytochromes would permit calculation of the proportion $b_{active}/b_{sluggish}$, a ratio which we equated to Y_{Ox}/Y_{red}. As the ratio c_{Ox}/c_{Red} under the same conditions was known, the difference in the midpotentials between Y and the c-type cytochromes could be estimated. The changes in the kinetic behaviour of cytochrome b were not due to variations in its apparent redox potential [3,7–12], since in these experiments cytochrome b was fully reducible (fig. 1).

Typical results of the experiments just described are shown in fig. 1. In the presence of antimycin and succinate, cytochromes $c + c_1$ were fully oxidized (not shown in the figure). Upon addition of ascorbate plus TMPD a new steady-state was achieved, and full reduction was obtained by addition of KCN (bottom curve, fig. 1). The steadystate of cytochromes $c + c_1$ under these conditions was independent of the presence of succinate. In a parallel experiment the same concentrations of ascorbate and TMPD were added to antimycin-inhibited ETP_H, and after the steady-state level of the c-type cytochromes has been reached (less than 5 sec), succinate was added and reduction of cytochrome b was followed (fig. 1, top). At 2°C the rate constants for the reduction of the active and the sluggish forms of cytochrome b were measured with KCN and antimycin inhibited ETP_H (not shown). The rate constants were 0.092 s^{-1} and 0.014 s^{-1} for the rapid and the slow phases, respectively. At any given ratio of Y_{Ox}/Y_{Red}, we expect the reduction of cytochromes b to be the sum of two parallel reactions with the appropriate rate constants. As shown in fig. 2, the decrease in absorbance at 563–575 nm followed biphasic kinetics. The second phase was typical of the reduction of sluggish cytochrome b, while the first phase was the

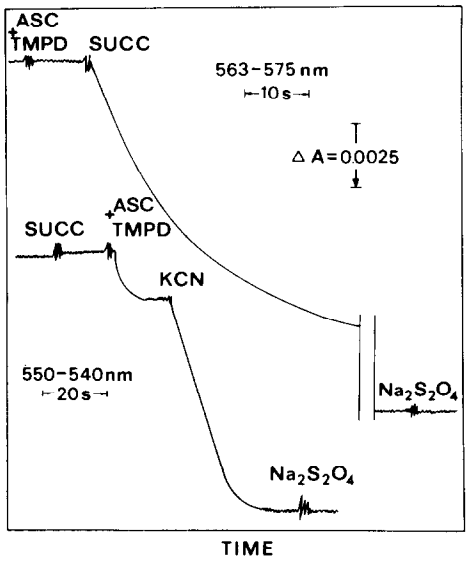


Fig. 1. The reduction of the b- and c-types cytochromes by succinate and ascorbate plus TMPD. ETP_H, washed and activated by malonate as described previously [1], were suspended to 2 mg/ml in 0.18 M sucrose, 5 mM $MgSO_4$, 50 mM Tris–acetate pH 7.4. Antimycin (2 nmol/mg) was added, followed by ascorbate (3mM), TMPD (60 μM) and succinate (6.7 mM) in the order shown in the figure. Full reduction of the c-type cytochromes was achieved by addition of KCN (2mM). Solid $Na_2S_2O_4$ was added to confirm maximal reduction. Temperature = 2°C.

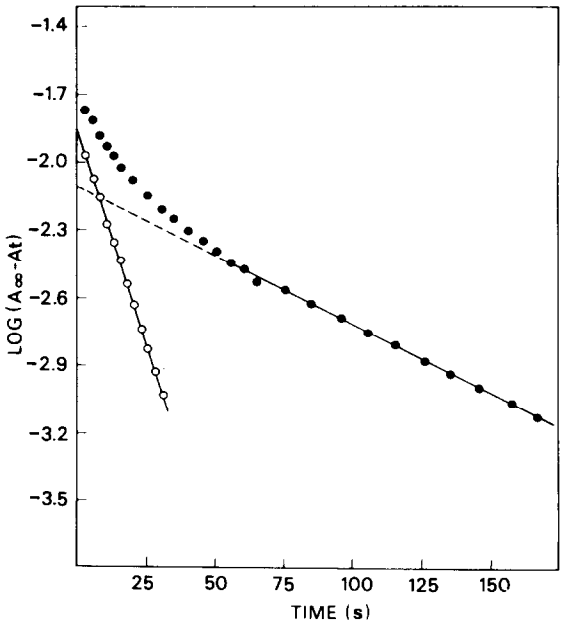


Fig. 2. Semi-logarithmic plot of the reduction of cytochrome b (563–575 nm), shown in fig. 1. The experimental conditions are described in fig. 1. (•) The experimental points taken from fig. 1; the rate constant of the slow reduction equals 0.014 s^{-1}. (○) The reduction of the active cytochrome b, calculated as detailed in the text; k=0.092 s^{-1}.

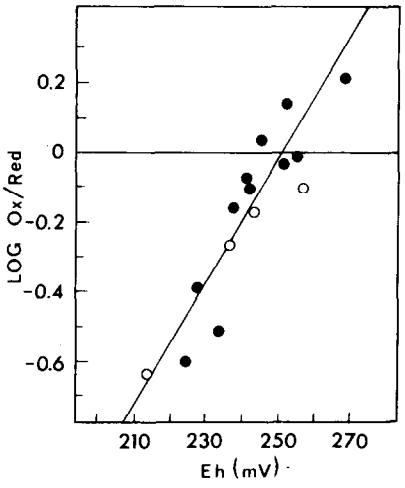
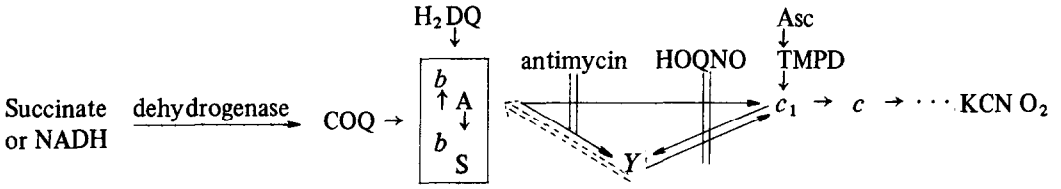


Fig. 3. Nernst plot for the reduction of *Y*. The experimental procedure is described in the text. (•) Normal ETP_H, (○) cytochrome *c*-depleted ETP_H. Temperature = 2°C.

sum of the rapid and the slow reactions. By extrapolating the line of the slow phase and measuring the differences between the observed values and the extrapolated line of the slow phase we could estimate the reduction rate of the rapidly reduced cytochrome *b*. These values, drawn in fig. 2 (open circles) fitted a first-order kinetics with k=0.092 s^{-1} – a value typical for active cytochrome *b* [1].

The amount of the active form of cytochrome *b* was estimated as the difference between the observed and extrapolated absorbancies (563–575 nm) at t=0. The rest of the *b*-type cytochromes were taken to be in the sluggish form. The experiments and kinetic analysis of the results, depicted in fig. 1 and 2, were repeated with varying TMPD concentrations (50–600 μM) which poised the reduction-level of the *c*-type cytochromes in the range of 28% to 71%. These experiments were repeated with four different batches of ETP_H, as well as with one batch of cytochrome *c*-depleted ETP_H. In all these experiments the rate constant of the second phase was 0.015 ± 0.003 s^{-1} and that estimated for the reduction of active cytochrome *b* was 0.09 ± 0.01 s^{-1}. For each experiment the apparent redox potential of the *c*-type cytochromes was calculated according to the Nernst equation (using a value of 2.30 ± 3 mV as the E'_m of the *c*-type cytochromes [13,14]. In fig. 3 these results were summarized; as can be seen the function log (Y_{Ox}/Y_{Red}) varied linearly with the poised potential, yielding E'_m = 2.52 ± 6 mV with n=1.0.

The results of studies with inhibitors indicate that the site of *Y* is on the oxygen side of the antimycin block [1]. In this work we have shown that the mid-potential of *Y* is higher than that of the *c*-type cytochromes. However in the presence of HOQNO, *Y* cannot be reduced by the *c*-type cytochromes [6]; therefore *Y* is not likely to be a carrier of the respiratory system, but rather, it may be a regulatory component – functionally located between cytochromes *b* and c_1. The mechansim suggested is depicted in the following scheme, where solid arrows represent redox reactions and dotted double arrows show the regulatory mechansim:



Y can be reduced either by the *c*-type cytochromes in an HOQNO sensitive reaction and probably by the *b*-type cytochromes in an antimycin sensitive reaction. When *Y* becomes reduced, it interacts with cytochrome *b*, converting the cytochrome to its sluggish form.

Very recently Lee and Slater [15] showed the existence of a new component having an EPR signal below 10°K. This component, located on the oxygen side of the antimycin block, is reducible by succinate and ascorbate plus phenazine-ethosulfate only under anaerobic conditions. This behaviour is not that expected of the '*X*' component, proposed by Rieske et al. [16,17], but it is compatible with what we project for *Y*. It is therefore possible that our *Y* component and the new species of Lee and Slater [15] may be the same.

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