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# ALKALI-INDUCED REDUCTION OF THE *b*-CYTOCHROMES IN PURIFIED COMPLEX III FROM BEEF HEART MITOCHONDRIA

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## SUMMARY

Approx. 40–50 % of the cytochrome *b* in purified Complex III is reduced by ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine or phenazine methosulfate at neutral pH. The remaining cytochrome *b*, including cytochrome *b*-565, is reduced by increasing the pH. The apparent p*K* for this reduction is between pH 10 and 11, and is more than two pH units higher than a similar alkali-induced transition in Mg-ATP particles. Alkali-induced reduction of cytochrome *b* occurs concomitantly with the exposure of hydrophobic tyrosine and tryptophan residues to a more hydrophilic environment. The relationship of these findings to the presence of a substrate accessibility barrier in Complex III is discussed.

## INTRODUCTION

Alkali-induced reduction of cytochrome *b* was first reported by Azzi and coworkers [1, 2] and Lee and Slater [3]. Azzi et al. [2] observed an inverse relationship between the amount of cytochrome *b* undergoing reduction with increased alkalization of the media and that reduced after subsequent addition of ATP. They concluded that alkalization affects the same cytochrome *b* as does ATP-induced energization, and thus mimics the energization reactions. Lee and Slater [3] favored the view that alkali removes a physical or kinetic barrier, allowing more rapid equilibration of substrate and cytochromes. The various arguments have been recently reviewed by Wikström [4].

In the present study it is demonstrated that the alkali-induced reduction of cytochrome *b* also occurs in highly purified Complex III. The apparent p*K* for reduction is, however, nearly two pH units higher than in mitochondria [1—3].

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; PMS, phenazine methosulfate.

## MATERIALS AND METHODS

Complex III was purified from beef heart mitochondria as described by Rieske et al. [5]. Mg-ATP submitochondrial particles were prepared from heavy beef heart mitochondria [6]. Experiments were carried out in either (A) 50 mM Tris · HCl, 167 mM sucrose or (B) 25 mM Tris/acetate, 50 mM glycine and 100 mM KCl as indicated in the text and figure legends. The latter buffer system was selected to minimize possible $K^+$ effects on protein conformation induced during titration of pH with KOH. Similar results were obtained in both buffers.

Changes in pH were monitored with a radiometric GK 2302C combination pH electrode. According to the manufacturer's data, the error due to $K^+$ is minimal (0.05 pH unit at a meter reading of 13.5 pH units in 200 mM $K^+$). Corrections for $K^+$ error were made at meter readings above pH 12 when media B was used. The pH was routinely adjusted by additions (1–10 $\mu$l) of 1 M KOH and the pH and absorbance changes were measured simultaneously. Subsequent additions of KOH were made only after the absorbance changes had stabilized. A complete titration curve of cytochrome *b* reduction or tyrosine ionization takes about 15–20 min. Titration of pH under anaerobic conditions were made in a cuvette designed for redox titrations [11], maintained under a flux of argon.

Cytochrome *b* was measured at 562 minus 575 nm (mM extinction = 20) and cytochrome $c_1$ at 554 minus 540 nm (mM extinction = 19) [7]. Tyrosinate anion was measured from the difference spectra: alkali pH minus neutral pH, using mM extinction coefficients of 11.1 at 243 nm or 2.3 at 295 nm [8]. All measurements were carried out using an Aminco-Chance dual wavelength spectrophotometer. Absolute spectra were continuously recorded under different conditions of titration, but adjustments for the baseline and calculation of difference spectra were often made manually at the end of each titration. This was done in order to eliminate the time required to readjust the baseline with the baseline compensator on the spectrophotometer, and, thus, to shorten the time of exposure of the protein to high pH.

## RESULTS

As reported earlier [9–11], the cytochromes of purified Complex III are similar to those in more intact membranes. Complex III contains a high potential ($E_{m\,7.2}$ = +93 mV), enzymatically reducible cytochrome *b*-562, and a low potential ($E_{m\,7.2}$ = −34 mV), chemically reducible cytochrome *b*-565 [11]. Fig. 1 shows, however, that after reduction of the *b*-cytochromes of Complex III, additional reduction is observed upon alkalinization of the media with KOH. The figure shows the spectrum of the cytochromes reduced by ascorbate plus phenazine methosulfate (PMS) at pH 7.5 (curve A) and those subsequently reduced after a pH jump from 7.5 to 11.3 (curve B). In this experiment approx. 40 % of the cytochrome *b* and all of the cytochrome $c_1$ is reduced by ascorbate plus PMS at pH 7.5. The spectrum of the remaining cytochrome *b* undergoing reduction in alkali is very broad and slightly red shifted, in accordance with the reduction of both cytochrome *b*-565 and a shorter wavelength cytochrome *b* [10]. In a few experiments a split peak at 560 and 564 nm has been observed.

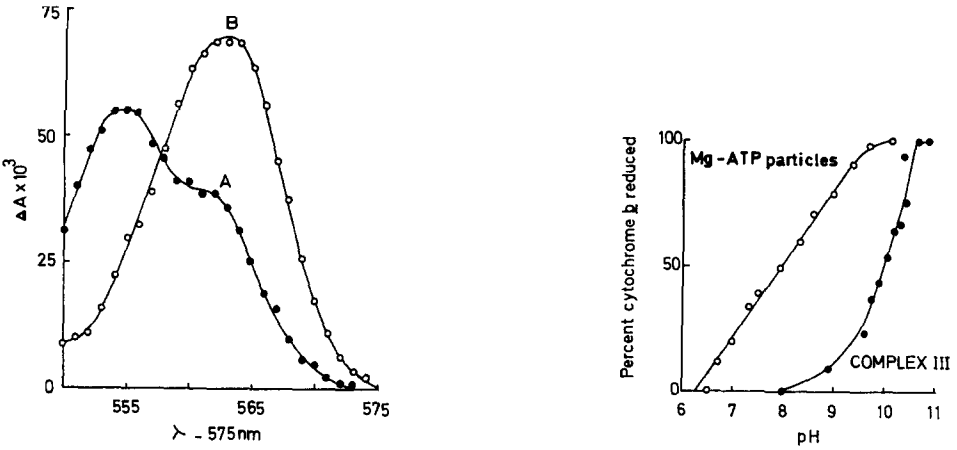


Fig. 1. Spectral analysis of the cytochromes of purified Complex III reduced at high pH. (A) Reduced by ascorbate plus PMS at pH 7.5. (B) Reduced after adjusting the pH from PH 7.5 to 11.3 with 1 M KOH. Curve B is the difference spectrum: reduction at pH 11.3 minus reduction at pH 7.5. The incubation mixture contained 50 mM Tris · HCl, 167 mM sucrose, 5 mM ascorbate and 5 $\mu$M phenazine methosulfate. Spectra were continuously recorded with the reference wavelength fixed at 575 nm.

Fig. 2. Reduction of cytochrome *b* in purified Complex III and Mg-ATP particles as a function of the pH. Complex III and Mg-ATP particles (in the presence of 1.67 mM KCN) were reduced by 5 mM ascorbate and 100 $\mu$M TMPD at pH 6.5 under a flux of Ar. The pH was increased by addition of Ar-flushed KOH, and reduction of cytochrome *b* measured at 562 minus 575 nm. The media contained 50 mM Tris and 167 mM sucrose.

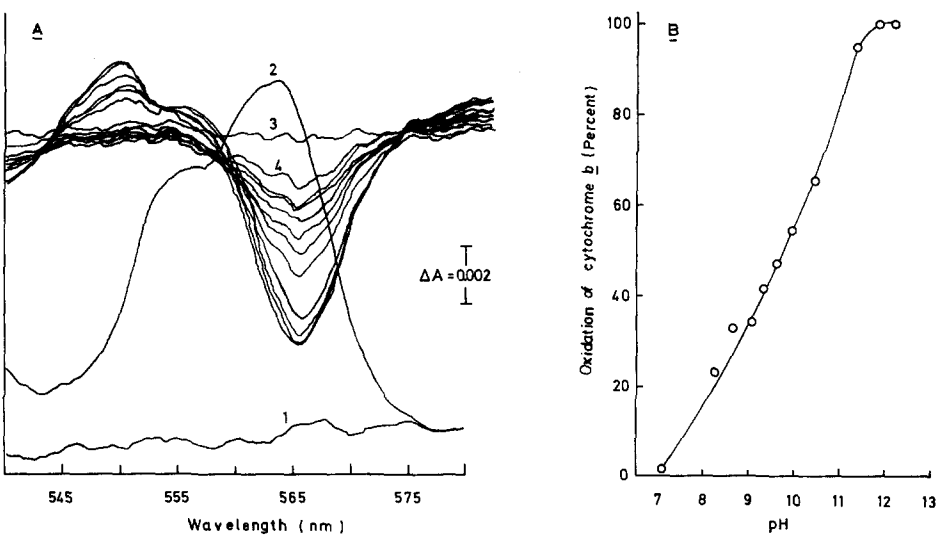


Fig. 3. Oxidation of the cytochromes in dithionite-reduced Complex III as a function of increasing pH. (A) Spectral analysis: curve 1, base line with oxidized Complex III in both cuvettes; curve 2, dithionite to sample cuvette; curve 3, baseline with dithionite-reduced Complex III in both cuvettes at pH 6.8; curve 4 plus the non-numbered curves, pH of sample cuvette increased from pH 6.8 by successive additions of 5 M KOH. (B) Plot of the experimental values for the oxidation of dithionite-reduced Complex III by increasing the pH. The reaction mixture contained 25 mM Tris/acetate, 50 mM glycine and 100 mM KCl, pH 6.8. The system was aerobic.

Fig. 2 compares the pH profiles for the alkali-induced reduction of cytochrome *b* in Mg-ATP particles and purified Complex III. Both preparations were first reduced with ascorbate plus TMPD at a pH value of 6.5. The reduction observed upon increasing the pH above these base levels was taken as the alkali-induced reduction of the cytochromes. It is quite clear that reduction of cytochrome *b* in Complex III occurs at a higher pH (between 10 and 11) than in Mg-ATP particles or mitochondria [1–3]. Reduction of cytochrome *b* can be reversed upon back titration with HCl. The significance of this titration is difficult to assess, however, since the reductant (ascorbate) is probably completely or partially destroyed at high pH, and acidification leads to oxidation of all cytochrome *b*, including that initially reduced with ascorbate plus TMPD or PMS at neutral pH.

Two factors influence, however, the extent to which cytochrome *b* becomes reduced upon alkalinization. The first is a pH-dependent autooxidation of cytochrome *b*-565, as shown for the dithionite-reduced Complex III in Fig. 3A. Under aerobic conditions oxidation is initiated at a lower pH (Fig. 3B) than is alkali-induced reduction (Fig. 2). Thus, under aerobic conditions, total reduction of cytochrome *b* is not always observed upon increasing the pH. The second factor affecting the extent of cytochrome *b* reduction measured at fixed wavelengths is a pH-dependent shift in the $\alpha$ band (Fig. 4). This shift occurs between pH 11 and 11.5, effecting only the upper part

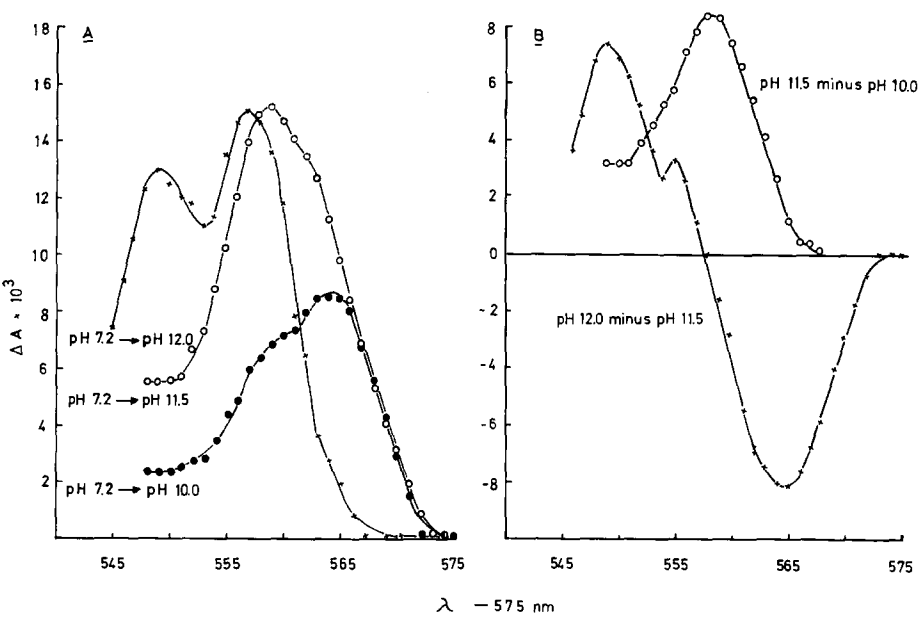


Fig. 4. Spectral changes induced in cytochrome *b* at high pH. Conditions were as in Fig. 3 except that 5 mM ascorbate and 150 $\mu$M TMPD were the reductants, and anaerobiasis was maintained by a constant flux of Ar. (A) Absolute spectra of cytochromes reduced by raising the pH. Approx. 50 % of the total cytochrome *b* was first reduced at pH 7.2 with ascorbate plus TMPD. A flat base line was adjusted and further reduction was initiated by increasing the pH. Though the data are expressed as reduction occurring above pH 7.2, no additional reduction was actually observed until pH 9.75 was obtained. (B) Difference spectra between the indicated pH values. Spectra were continuously recorded with the reference wavelength fixed at 575 nm.

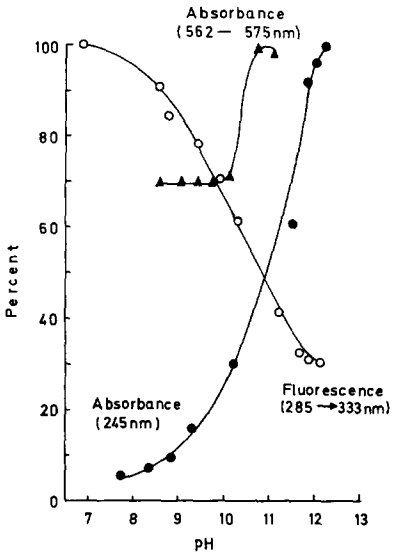


Fig. 5. Effects of pH on the reduction of cytochrome *b*, the ultraviolet absorption and fluorescence of Complex III. ▲, reduction of cytochrome *b* at 562 minus 575 nm; ●, increase in tyrosine absorbance at 245 nm; and ○, quenching of tryptophan fluorescence excited at 285 nm and read at 330 nm. The reaction media contained 25 mM Tris/acetate, 50 mM glycine and 100 mM KCl, pH 6.8. The pH was increased with KOH. Reduction of cytochrome *b* was carried out in the presence of 5 mM ascorbate and 100 $\mu$M TMPD, under aerobic conditions.

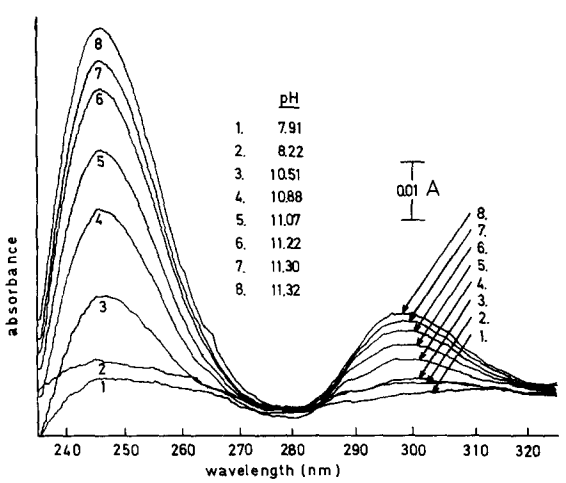


Fig. 6. Effects of pH on the ionization of tyrosine in Complex III. The reference and sample cuvettes both contained 167 mM sucrose, 50 mM Tris · Cl, pH 7.5, and 0.2 mg Complex III protein. The pH was then increased in the sample cuvette by addition of KOH.

of the titration curves. Both the long and short wavelength *b*-cytochromes are influenced by high pH. The short wavelength cytochrome is either blue shifted, or an additional amount of cytochrome *b*-558 becomes reduced when the pH is raised from 10.0

to approx. 11.5 (Figs 4A and 4B). At pH 12.0 the $\alpha$ peak of cytochrome *b*-558 is shifted to 557 nm and that of cytochrome *b*-565 is shifted to 548–549 nm. Similar spectral shifts are observed in both dithionite- (Fig. 3A) and ascorbate plus TMPD-reduced (Fig. 4B) samples, and cannot be considered artifacts due to the effects of high pH on the spectral properties of the reductants.

Fig. 5 shows that the alkali-induced reduction of cytochrome *b* in Complex III is associated with an increase in the ultraviolet absorbance of the complex due to ionization of the hydroxyl group of tyrosine, and with a decrease in fluorescence at 330 nm due to quenching of tryptophan fluorescence. The emission spectrum of tryptophan is shifted from 330 nm to 345 nm (not shown) during transition from pH 7.5 to 12.5, indicating the movement of tryptophan from a hydrophobic to more hydrophilic environment [12]. The high pH values required to ionize tyrosine suggest that most of the residues in Complex III are buried in the hydrophobic portion of the complex [8]. The reaction was carried out under aerobic conditions, thus accounting for the small amount of cytochrome *b* reduced. The total tyrosine content of Complex III (about 25 mol/mol of cytochrome *b* heme) was determined from the difference spectra: pH 12.5 minus pH 7.5 after titrating with KOH as shown in Fig. 6.

## DISCUSSION

Azzi and Santato [1] suggested that alkali-induced reduction of cytochrome *b* occurs only after removal of a proton from oxidized cytochrome *b* as shown in Eqn 1.

$$\text{cytochrome } b_{ox}^{\cdot H^+} \longrightarrow \text{cytochrome } b_{ox} + H^+ \quad (1a)$$

$$\text{cytochrome } b_{ox} + e^- \longrightarrow \text{cytochrome } b_{red} \quad (1b)$$

However, it is clear from the pH dependency (−60 mV/pH unit) of the half-reduction potentials of the *b*-cytochromes [13, 14], that oxidation of cytochrome *b* is also accompanied by proton release

$$\text{cytochrome } b_{red}^{\cdot H^+} \longrightarrow \text{cytochrome } b_{ox} + e^- + H^+ \quad (2)$$

It is obvious that the reaction shown in Eqn 1 cannot involve the same protonated group, or perhaps not even the same peptide, as in Eqn 2. The explanation offered by Lee and Slater [3] and Wikström [4] for the alkali-induced reduction of cytochrome *b* therefore seems to be applicable. These investigators have proposed that alkali leads to the removal of a physical or kinetic barrier (Eqn 1) allowing substrate to equilibrate more rapidly with cytochrome *b*. This barrier prevents succinate and NADH from reducing cytochrome *b*-565 [15] in mitochondria and submitochondrial particles, and can be overcome by energization in the presence of ATP [15], or by antimycin [16]. It is argued that reduction at high pH is not due to increasing differences in the redox potentials of the components involved [3, 4] since (1) the potential of NADH should itself be low enough to completely reduce cytochrome *b*-565 even at neutral pH and (2) increasing pH should lower the potential of both the substrate and cytochrome *b* proportionally, and should not, therefore, lead to a difference between the half-reduction potentials of the two.

The present study shows that alkali-induced reduction of cytochrome *b* can also be observed in purified Complex III, a particle much simpler with respect to its

composition than the intact inner membrane. The response differs from that in mitochondria in two respects, (1) the apparent p$K$ for reduction of cytochrome *b* is nearly two pH units higher in the isolated complex, and (2) PMS does not release the effect [3], but rather participates in the reduction reaction.

The change in the apparent p$K$ is of particular interest. Since the kinetic, spectral and redox properties of purified Complex III remain essentially unaltered from those in more intact membranes [10, 11], we can assume that the heme environments of the two *b*-cytochromes have not been drastically disturbed during the purification. If this is correct, then the change in the apparent p$K$ for reduction of cytochrome *b* must be associated with the loss of additional components (proteins or lipids) which, in more intact membranes, are able to transmit environmental changes due to changing proton concentrations to the cytochrome *b* molecule. Removal of these components still leaves us with a barrier in Complex III, as the alkali-induced reduction of cytochrome *b* is observed. This may be explained if, (1) in preparing Complex III, we have created a new barrier without actually disturbing the heme environment, or (2) the barrier in purified Complex III more accurately reflects the environment of the heme groups as they exist in the membrane, uninfluenced by "mediator components".

It is clear from the present studies that to overcome the barrier in Complex III requires a physical change in the complex which is associated with the ionization of hydrophobic tyrosine groups and the exposure of tryptophan residues to more hydrophilic environments. However, presently it is not possible to state whether tyrosines are directly involved in the redox reactions of the cytochromes [17, 18] or merely reflect a conformational change in the complex.

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