BBA Report

BBA 41203

Biochemical and biophysical studies on cytochrome aa_3. VII. The effect of cytochrome c on the oxidation-reduction potential of isolated cytochrome aa_3

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(Received February 11th, 1972)

SUMMARY

1. In the absence of cytochrome c, the haem groups of cytochrome aa_3 titrate as indistinguishable identities, each having E'_o = 280 mV and n = 1.

2. In the presence of cytochrome c, 45% of the absorbance change on reduction at 605 nm is due to a high-potential component with E'_o = 335-360 mV, n = 1.2 and 55% to a low-potential component with E'_o = 200-250 mV, n = 1.0. At 445 nm both components contribute equally to the difference spectrum (reduced *minus* oxidized).

Determinations of the midpoint potential of cytochrome aa_3 (EC 1.9.3.1) both in isolated and in particulate systems have been carried out by two different techniques: (a) comparative measurements, where the potential has been determined in combination with and relative to cytochrome c, and (b) absolute measurements, where the absorbance and potential changes upon reduction have been simultaneously monitored both spectrophotometrically and potentiometrically.

Comparitive measurements[1-3] with isolated cytochrome aa_3 and cytochrome c, applying the equation

$$\frac{60}{n} \log \frac{[aa_3 \text{ (oxidized)}]}{[aa_3 \text{ (reduced)}]} = \Delta E'_o + 60 \log \frac{[c^{3+}]}{[c^{2+}]}$$

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have led to midpoint potentials for cytochrome aa_3 in the range from 270 to 290 mV, with values of about 0.5. Absolute measurements with isolated cytochrome aa_3 *plus* cytochrome c[4] and in mitochondria and submitochondrial particles[5-8], applying the equation

$$E = E'_o + \frac{60}{n} \log \frac{[aa_3 \text{ (oxidized)}]}{[aa_3 \text{ (reduced)}]}$$

have led to the same result.

Wilson and co-workers[6-8] have shown that in particulate systems these values are the resultant of a high-potential haem a component (E'_o = 340–395 mV, n = 1) and of a low-potential one (E'_o = 190–222 mV, n = 1). The high-potential species was some tentatively identified as cytochrome a_3, and the low-potential species as cytochrome a.

In the absence of cytochrome c, van Gelder[9] found a linear increase of $A_{605\,nm}$ and $A_{445\,nm}$ by titration of isolated cytochrome aa_3 with NADH and catalytic amounts of phenazine methosulphate. This suggests that the two haem groups either have the same midpoint potential or the same extinction coefficients.

We therefore investigated the effect of cytochrome c on the midpoint potential of highly purified cytochrome aa_3, making use of both experimental techniques mentioned above.

Part of this work has been mentioned by Slater[10] in a preliminary form.

Horse-heart cytochrome c was isolated according to Margoliash and Walasek[11], and beef-heart cytochrome aa_3 according to Fowler *et al.*[12] and MacLennan and Tzagoloff[13] (see also ref. 14). The final cytochrome aa_3 preparation contained 4.5–5 μmoles cytochrome aa_3 (9–10 μmoles haem a) per g protein. The extinction coefficients (reduced *minus* oxidized) used for cytochrome c and cytochrome aa_3 were 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm (ref. 15) and 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm (ref. 9), respectively.

Cholic acid was obtained from British Drug Houses and recrystallized from ethanol before use; Tween-80 and phenazine methosulphate were from Sigma, other reagents were mainly British Drug Houses, Analar grade. NADH, grade 2, came from Boehringer. NADH concentrations were calculated with ϵ = 6.22 $mM^{-1} \cdot cm^{-1}$ at 340 nm (refs 16 and 15).

Spectrophotometric measurements were performed on Cary-14 or Cary-17 spectrophotometers. Potentials were measured to ± 1 mV with a Philips PW9408 digital pH/mV meter. Platinum and calomel electrodes were routinely calibrated before and after each titration with quinhydrone and standard buffer, and the whole titration assembly was periodically tested by titration of 2,6-dichlorophenol indophenol. The titration vessel was kept anaerobic by continuous flushing with nitrogen gas made by evaporation of liquid nitrogen.

Comparative determinations were carried out by anaerobic titration of a mixture of cytochrome aa_3 and cytochrome c with NADH in a Thunberg cuvette equipped with a hollow stopper. The cuvette was made anaerobic by repeated

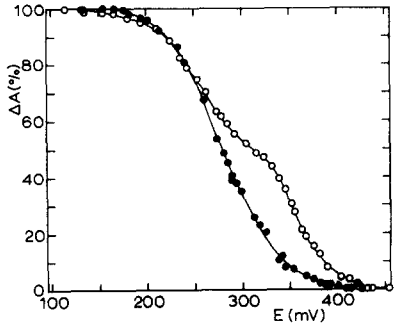
evacuation and flushing with argon[17]. After anaerobiosis was reached, a 3-fold excess of NADH was tipped in from the stopper. Complete reduction (in the absence of phenazine methosulphate) was achieved in 6–8 h. Spectra were taken in the range from 700 to 330 nm at 10-min intervals. From the decrease in A_{340nm} the NADH consumption in electron-equivalents was related to the absorbance changes at 445, 550 and 605 nm.

Titrations of cytochrome aa_3 in the absence of cytochrome *c* were carried out in separate anaerobic Thunberg cuvettes with phenazine methosulphate as a catalyst and known amounts of NADH[9].

All experiments were carried out at 21–23 °C in 100 mM potassium phosphate (pH 7.0–7.2) with 0.5% cholate or Tween-80. No difference was found between these two detergents.

The potentiometric titrations of isolated cytochrome aa_3 (●——●) and of the mixture of stoicheiometric amounts (on haem basis) of cytochrome aa_3 and cytochrome *c* (○——○) are shown in Fig. 1, where the redox state is plotted against the redox potential. The titration curve of cytochrome aa_3 in the absence of cytochrome *c* fits very closely to a theoretical $n = 1.0$ line, as can be seen from Fig. 2 (●——●). A midpoint potential $E'_o = 280$ mV is obtained from both Figs 1 and 2. It should be emphasized that the titration of isolated cytochrome aa_3 can easily be disturbed by traces of oxygen, giving rise to the formation of 'oxygenated' cytochrome aa_3 which interferes with the measurements[17].

The titration of the stoicheiometric mixture of cytochrome aa_3 and cytochrome *c* shows a large deviation from the $n = 1.0$ curve in the high-potential region (see Fig. 1), and a smaller deviation in the low-potential region. The best fit in the 200–320 mV range is obtained by a line with $E'_o = 300$ mV, $n = 0.5$, as can be seen from Fig. 2 (○——○). It is



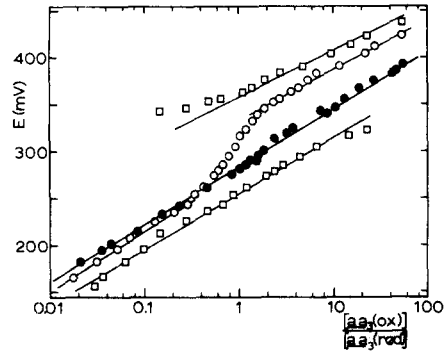


Fig. 1. Potentiometric titration at 605 nm of cytochrome aa_3 (15 μM) in the absence (●——●) and presence (○——○) of cytochrome *c* (30 μM). The line in the presence of cytochrome *c* has been corrected for the contribution of cytochrome *c* to ΔA_{605nm}. Mediators: 20 μM phenazine methosulphate, 20 μM diaminodurene and 120 μM $K_3Fe(CN)_6$. Reduction was brought about by 3-μl additions of 10 mM NADH to a reaction volume of 10 ml. The ordinate represents the percentage of the difference in absorbance between fully reduced and fully oxidized.

Fig. 2. Semi-logarithmic plot of the potentiometric titration of cytochrome aa_3 in the absence (●——●) and presence (○——○) of cytochrome *c*, and the resolved lines in the presence of cytochrome *c* (□——□). The points have been calculated from the experiment depicted in Fig. 1.

clear that the titration curve for the stoicheiometric mixture in Fig. 1 is the resultant of two superimposed curves, one representing a high-potential component which accounts for about 45%, and one a low-potential component accounting for about 55% of the total absorbance change. The resolved curves give one component with E'_0 = 360 mV, n = 1.2 and one with E'_0 = 250 mV, n = 1.0 (Fig. 2).

The comparative titration of the mixture of cytochrome aa_3 and cytochrome c is given in Fig. 3, where the abscissa represents electron-equivalents taken up by the mixture in the presence of an excess of NADH. To be sure that these results represent no significant departure from equilibrium, the reductions were also carried out (not shown) in separate cells with varying small amounts of NADH plus a trace of phenazine methosulphate for its rapid consumption. Both techniques gave the same results. Fig. 3 shows that the reduction of cytochrome c (550 nm) initially lags behind that of part of the cytochrome aa_3 and later on precedes that of the other part of the cytochrome aa_3. As in the potentiometric titration, the curves at 445 and 605 nm are the resultant of two superimposed curves, one contributing 45% of the total absorbance difference at 605 nm and 50% at 445 nm, and the other 55% at 605 nm and 50% at 445 nm.

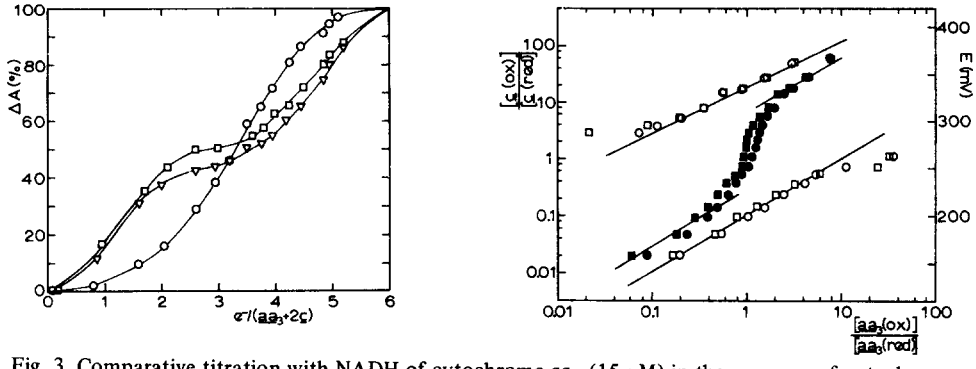


Fig. 3. Comparative titration with NADH of cytochrome aa_3 (15 μM) in the presence of cytochrome c (30 μM). □—□, 445 nm (aa_3); ○—○, 550 nm (c); ▽—▽, 605 nm (aa_3). The lines have been corrected for the contribution of cytochrome aa_3 to $\Delta A_{550\,nm}$ and for the contribution of cytochrome c to $\Delta A_{445\,nm}$ and $\Delta A_{605\,nm}$.

Fig. 4. Double logarithmic plot of $[c(ox)]/[c(red)]$ against $[aa_3(ox)]/[aa_3(red)]$. The ordinate has been recalculated to a potential scale with E'_0 (c) = 260 mV. ■—■, 445 nm; ●—●, 605 nm, □—□, 445 nm, resolved lines; ○— ○, 605 nm, resolved lines. The points have been calculated from the experiment shown in Fig. 3.

When a potential scale is calculated from the degree of reduction of cytochrome c, taking E'_0 for cytochrome c at 260 mV, Fig. 4 is obtained. This shows two components, one with E'_0 = 335 mV, n = 1.2 and one with E'_0 = 200 mV, n = 1.0. It may be noted that the unresolved curve in Fig. 4 suggests E'_0 = 270 mV, n = 0.5.

The titration of cytochrome aa_3 in the absence of cytochrome c (Fig. 5) shows a nearly straight line up to complete reduction. This suggests, in agreement with the direct potentiometric measurement described above, that the two haem a groups have the same midpoint potential.

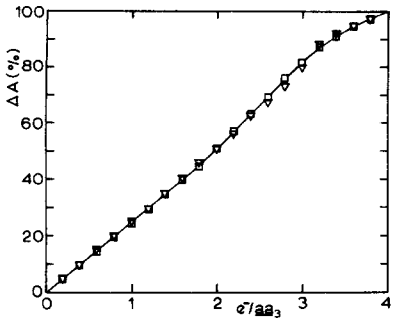


Fig. 5. Titration of cytochrome aa_3 with NADH and catalytic amounts of phenazine methosulphate in the absence of cytochrome *c*. □—□, 445 nm; ▽—▽, 605 nm. 10-40 μM cytochrome aa_3, 0.1-0.4 μM phenazine methosulphate. The points are the average of 10 experiments.

The potentiometric titration of cytochrome aa_3 in the absence of cytochrome *c* shows that only one haem *a* species is titrated. As cytochrome aa_3 contains a cytochrome *a* and a cytochrome a_3 moiety, the obvious conclusion is that in the absence of cytochrome *c*, cytochrome *a* and cytochrome a_3 have the same midpoint potential.

Our results with isolated cytochrome aa_3 *plus* cytochrome *c* are close to those of cytochrome aa_3 in particulate systems[5-8]. In the presence of cytochrome *c*, both the potentiometric and the comparative titration show the existence of a high-potential and a low-potential species. These same species and potentials are found when only 5% of the stoicheiometric amount of cytochrome *c* is present. The $n = 0.4$-0.6 lines in the 200-320 mV range in Figs 2 and 4 explain the $n = 0.5$ value previously found with isolated cytochrome aa_3 in the presence of cytochrome *c*[1-3,8] or in mitochondria[4].

The presence of two haem *a* components in isolated cytochrome aa_3 *plus* cytochrome *c* and in particulate systems can be explained in two ways: either a high-potential cytochrome aa_3 and a low-potential cytochrome aa_3 are present, as proposed by Nicholls (personal communication), or the high-potential haem *a* is identical with cytochrome a_3 and the low-potential haem *a* with cytochrome *a*, as proposed by Wilson and Dutton[5]. The latter possibility is favoured by our finding (not shown) that the high-potential component shows a single Soret band on reduction, whilst the low-potential one has a split Soret band, which is thought to be characteristic of cytochrome *a*[18,19].

We see as an attractive possibility that the two haem *a* groups in isolated cytochrome aa_3 are equivalent (*cf.* refs 20,21). Cytochrome *c*, or ligands induce a conformational change in the cytochrome aa_3 molecule, such as to differentiate between the two haem *a* groups by converting one of them to cytochrome *a* and the other one to cytochrome a_3. The effect of less than stoicheiometric amounts of cytochrome *c* can be explained by a relaxation of this conformation, which is slow in comparison with the rapid binding[22] ($k = 4 \cdot 10^7$ $M^{-1} \cdot s^{-1}$) and dissociation[23] ($k = 1200$ s^{-1}) of cytochrome *c*.

The distinction between the contributions of cytochrome a_3 (20%) and cytochrome *a* (80%) to the difference spectrum (reduced *minus* oxidized) at 605 nm

determined in the presence of cyanide[9,24,25] might be explained by a 25% increase of the absorbance of the ferric cytochrome a_3 cyanide compound following reduction of cytochrome *a* or by an influence of cyanide on the spectrum of cytochrome *a*.

We wish to thank Prof. E.C. Slater for his stimulating interest and for valuable discussion. This work was supported in part by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

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