# CYTOCHROME *c*–CYTOCHROME $aa_3$ COMPLEX FORMATION AT LOW IONIC STRENGTH STUDIED BY AQUEOUS TWO-PHASE PARTITION

Lars Chr. PETERSEN
*Institute of Biochemistry, Odense University, Campusvej 55, DK-5230 Odense M, Denmark*

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## 1. Introduction

Cytochrome *c* interacts with mitochondria and purified cytochrome *c* oxidase to form complexes (reviewed [1–3]). A complex stable to chromatography and to ultracentrifugation is obtained with purified oxidase at low ionic strength [4–7]. One site per oxidase molecule (per 2 heme *a*) [4–8] with a very high affinity for cytochrome *c* is involved in the formation of this stable complex, but also sites with lower affinities may exist together with the high affinity site [9–11].

Despite recent progress in the knowledge about the cytochrome *c*–cytochrome $aa_3$ interaction at the molecular level [1,7,11–13] many problems about the functional aspects remain.

In this communication the poly(ethylene glycol)/dextran water two-phase system [14,15] has been used to study some binding properties of the high affinity site on cytochrome oxidase. This system provides a convenient assay for the complex formation. It is confirmed that the stoichiometry of the complex is 1:1, and it is shown that the affinity for ferri-cytochrome *c* is three times higher than for ferro-cytochrome *c*. The values of the dissociation constants measured by means of the two-phase partition assay are in agreement with values obtained from measurements of cytochrome *c* oxidation kinetics.

## 2. Materials and methods

Cytochrome *c* oxidase was prepared from beef heart by the method in [16]. Cytochrome *c* and poly(ethylene glycol) mol. wt 6000 was from Sigma. Dextran T500, mol. wt 500 000 was from Pharmacia Fine Chemicals.

The two-phase system consisted of 7% w/w dextran, 4.4% w/w poly(ethylene glycol), 0.5% Tween-80, 0.5% digitonin, 0.1% sodium cholate, and potassium phosphate buffer, pH 7.4, at 25°C. The detergents were added to minimize precipitation of oxidase at the interphase which was otherwise a serious problem. After mixing, the samples were centrifuged 10 min at 3000 rev./min in a clinical centrifuge to speed up phase separation. Samples were taken from the top and bottom phase and diluted twice before the concentration of cytochrome *c* and cytochrome $aa_3$ was measured in a Cary 118 C spectrophotometer, using the mM extinction coefficients $\epsilon_{550-540}$ (reduced–oxidized) = 21 and $\epsilon_{605-630}$ (reduced–oxidized) = 27.

Cytochrome *c* oxidation was measured following the decrease in $A_{550}$ or $A_{415}$ after addition of oxidase to medium containing reduced cytochrome *c*. The spectrophotometer was interfaced to a mini computer system for data collection. Values of observed first order constants were calculated from semi-logarithmic plots drawn by the computer.

## 3. Results

Aqueous phase partition systems for the separation and analysis of biological macromolecules and cell particles have been developed [14,15]. One potential application of such phase partition is in the

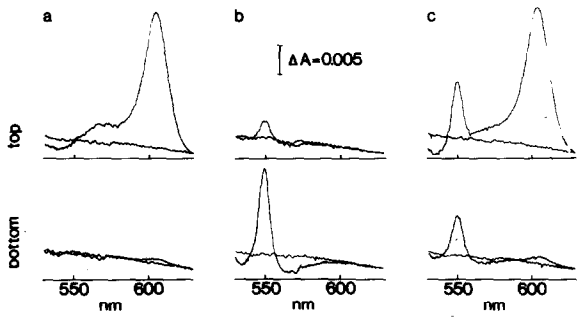


Fig.1. Partition of cytochrome $aa_3$ and cytochrome $c$ in the poly(ethylene glycol)/dextran two-phase system. The two-phase system was prepared as in the text and contained 20 mM potassium phosphate, pH 7.4. Prior to phase separation was added: (a) 1 μM cytochrome $aa_3$; (b) 1 μM cytochrome $c$; (c) 1 μM cytochrome $aa_3$ plus 1 μM cytochrome $c$. The reduced–oxidized difference spectrum of the top and bottom phases are shown.

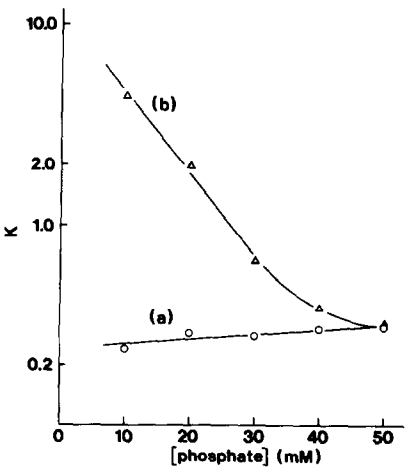


Fig.2. Partition of cytochrome $c$ at various concentrations of potassium phosphate. Various concentrations of phosphate, pH 7.4, were added to the two-phase system. The cytochrome $c$ concentration ratio top/bottom, equal to $K$ is plotted on a logarithmic scale as a function of the phosphate concentration. (a) 1 μM cytochrome $c$; (b) 1 μM cytochrome $c$ plus 1 μM cytochrome $aa_3$.

study of protein–protein interaction [15,17]. Figure 1 shows the partition of cytochrome $aa_3$ (fig.1a) and cytochrome $c$ (fig.1b) in the two-phase system formed after mixing of 4.4% w/w poly(ethylene glycol) and 7.0% w/w dextran. Cytochrome $aa_3$ has a high affinity for the poly(ethylene glycol) rich top phase (partition coefficient, $K$=20), whereas cytochrome $c$ has the highest affinity for the dextran rich bottom phase ($K$=0.275). Also shown (fig.1c) is the partition of cytochrome $aa_3$ plus cytochrome $c$ added in stoichiometric amounts. Cytochrome $aa_3$ is little affected by the presence of cytochrome $c$, however the fact that the partition of cytochrome $c$ is highly sensitive to the addition of cytochrome $aa_3$ is an indication of intermolecular interaction.

Figure 2 shows the partition of cytochrome $c$ as a function of the phosphate concentration when cytochrome $c$ plus cytochrome $aa_3$ are added to the two-phase system in stoichiometric amounts (curve b). Also shown (curve a) is the partition of cytochrome $c$ in the absence of cytochrome $aa_3$. The figure shows that the interaction is strongest at low phosphate concentration and that the interaction is not detectable by this method at $\gtrsim$ 40 mM phosphate.

According to [15] it is in principle possible to determine the binding constant describing the interaction between two molecules from 3 measurements of the partition such as those in fig.1. A method based on measurements at several concentrations of cytochrome $c$ but at a fixed concentration of cytochrome $aa_3$ gives a more accurate determination of the binding constant and in addition the stoichiometry of the complex formation can be obtained. The data from a series of such partition experiments at 20 mM potassium phosphate, pH 7.4, is shown in fig.3. Because of the unfavourable partition of cytochrome $aa_3$ (fig.1) the amount of complex-bound cytochrome $c$ in the bottom phase is negligible. This means that the concentration of free cytochrome $c$ in the top phase can be calculated from the concentration of free cytochrome $c$ in the bottom phase $[C_t^{free}] = K[C_b]$ where $K$ is the partition coefficient in the absence of added cytochrome $aa_3$. Thus the concentration of bound cytochrome $c$ in the top phase is given by:

$$[C_t^{bound}] = [C_t] - K[C_b]$$

where $[C_t]$ is the total concentration of cytochrome $c$ in the top phase. The plot of $([C_t]-K[C_b])/K[C_b]$ versus $[C_t]-K[C_b]$ shown in fig.3 is therefore equivalent to a

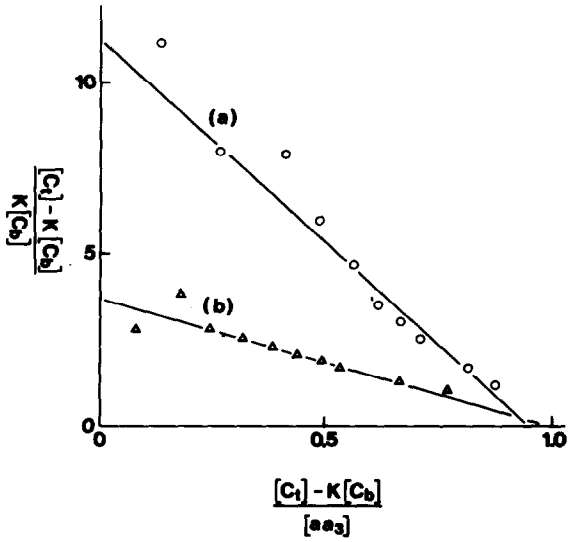


Fig.3. Scatchard plot of cytochrome *c* binding by cytochrome $aa_3$. $[C_t]$ and $[C_b]$ is the total concentration of cytochrome *c* in the top and bottom phase, respectively. *K* is the partition coefficient of cytochrome *c* in the absence of cytochrome $aa_3$. The plot of $([C_t]-K[C_b])/K[C_b]$ versus $[C_t]-K[C_b]$ correspond to a plot of $[C^{bound}]/[C^{free}]$ versus $[C^{bound}]$ (see text). Cytochrome *c* at various concentrations (0.2–4 μM) and cytochrome $aa_3$ (1 μM) were added to the two-phase system (20 mM potassium phosphate, pH 7.4) prior to phase separation. The concentration of cytochrome $aa_3$ in the top phase was 1.75 μM in all tubes. (a) No further additions; (b) plus 5 mM potassium ascorbate and 1 mM KCN.

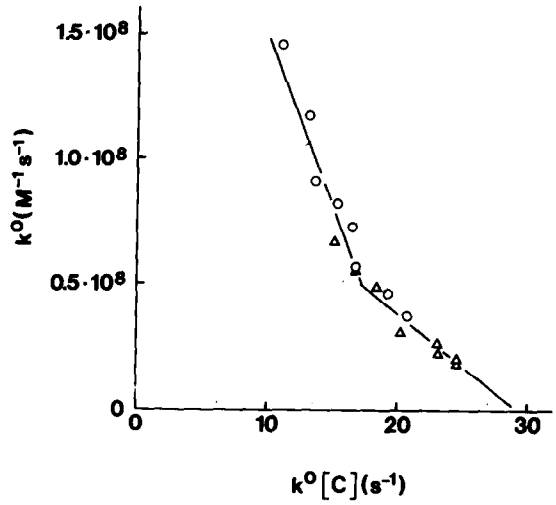


Fig.4. Eadie-Scatchard plot for cytochrome *c* oxidation catalyzed by cytochrome $aa_3$. In the plot of $k^o$ versus $k^o \cdot [C]$, equivalent to a *V/S* versus *V* plot, $k^o$ is the observed first order constant for cytochrome *c* oxidation divided by the concentration of cytochrome $aa_3$ and [C] is the concentration of ferro- plus ferri-cytochrome *c*. The reaction was followed at 550 nm or 415 nm. Additions: 20 mM potassium phosphate; 0.5% Tween-80; 0.1% cholate, pH 7.4. (○) 0.075–0.375 μM cytochrome *c*; 1 nM cytochrome $aa_3$. (△) 0.225–1.5 μM cytochrome *c*; 3 nM cytochrome $aa_3$

Scatchard plot [18]. In one type of experiment (fig.3, curve a) the interaction between ferri-cytochrome *c* and oxidized cytochrome $aa_3$ is investigated, and in the other (curve b) the interaction between ferro-cytochrome *c* and cyano-cytochrome $aa_3$. In both cases the stoichiometry of the cytochrome *c*–cytochrome $aa_3$ complex is 1.0, in accordance with [4–8], furthermore the figure clearly shows that the binding of ferri-cytochrome *c*, $K_d$ = 0.13 μM, is considerably stronger than the binding of ferro-cytochrome *c*, $K_d$ = 0.35 μM (20 mM potassium phosphate, pH 7.4).

Because the binding constant as well as the constant describing the steady-state kinetics of the cytochrome *c* oxidase reaction vary considerably with changes in the environment (e.g., the ionic strength of the medium) [2,6,19], it is of interest to determine the app. $K_m$ for cytochrome *c* under conditions similar to those used in the measurements of the binding constant.

Figure 4 shows the steady-state kinetics of the cytochrome *c* oxidation in a medium consisting of 20 mM potassium phosphate, pH 7.4, 0.5% Tween-80, 0.1% cholate. The reaction was strictly first order at the concentrations of cytochrome *c* tested (0.075–1.5 μM). The observed first order constant divided by the concentration of cytochrome $aa_3$, $k^o$, is plotted as a function of $k^o \cdot [C]$ where [C] is the concentration of ferro-cytochrome *c* plus ferri-cytochrome *c*. This plot is analogous to an Eadie-Scatchard plot of *V/S* versus *V*. The plot is non-linear in accordance with [7,10,11]. Two app. $K_m$ values of 0.075 μM and 0.25 μM are obtained from the slope of the straight lines shown in fig.4.

## 4. Discussion

The interaction between cytochrome *c* and purified cytochrome $aa_3$ can be followed in a direct manner by aqueous two-phase partition. It is shown (fig.3) that both reduced and oxidized cytochrome *c* forms a 1:1 complex with cytochrome $aa_3$ and it can be concluded that oxidized cytochrome *c* is more firmly bound to the high affinity site than reduced cytochrome *c*. These results confirm previous suggestions based on direct binding studies with mitochondria [20,21] and the difference in the dissociation constant between reduced and oxidized cytochrome *c* corresponds to a difference in the apparent redox potential between free and bound cytochrome *c* of 30 mV which agrees with the values obtained in [22].

The cytochrome *c* binding pattern observed here and in [20–22] is difficult to reconcile with any of the common mechanisms proposed to account for the steady-state kinetics of the oxidase reaction. This is because the assumption that the oxidase has the same affinity for ferro- as for ferri-cytochrome *c* [23] is the most plausible explanation offered so far [2,10, 11,24] to account for the fact that the cytochrome *c* oxidation catalyzed by the oxidase is strictly first order even at cytochrome *c* concentrations several orders of magnitude higher than the app. $K_m$.

The dilemma is not easily solved but the results obtained in fig.4 may suggest that at low turnover rates, cytochrome *c* in the enzyme substrate complex is in the oxidized form. It is possible that at high turnover rates the oxidase switches to another state where only the complex with ferro-cytochrome *c* needs consideration in the description of the steady-state kinetics.

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