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Biochimica et Biophysica Acta 1321 (1997) 238–242

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

# Binding of MOA-stilbene to the mitochondrial cytochrome $bc_1$ complex is affected by the protonation state of a redox–Bohr group of the 'Rieske' iron–sulfur protein

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Received 1 May 1997; revised 19 June 1997; accepted 25 June 1997

**Abstract**

MOA-stilbene is a specific inhibitor of the ubihydroquinone oxidation center (center P or o) of cytochrome $bc_1$ complex. Binding of this inhibitor does not require the 'Rieske' iron–sulfur protein, but is affected by the redox-state of the cytochrome $bc_1$ complex. We have analyzed the pH dependence of the apparent dissociation constant for MOA-stilbene. A 2.5 fold change in affinity between pH 6.0 and 9.5 was observed for oxidized bovine cytochrome $bc_1$ complex. The pH profile could be simulated by assuming a single protonable group with $pK_A = 7.7$. This $pK_A$ was not observed after partial or complete reduction of the enzyme or after removal of the iron–sulfur protein. We conclude that this protonable group was identical to the redox-Bohr group with the same $pK_A$ that has been reported to be associated with the 'Rieske' iron–sulfur cluster.

Fully reduced cytochrome $bc_1$ complex exhibited an additional binding site for MOA-stilbene. As this second binding site was abolished by the center P inhibitor stigmatellin, but not by antimycin, an inhibitor of ubiquinone reduction at center N, we conclude that it is also located at center P. © 1997 Elsevier Science B.V.

*Keywords:* Cytochrome $bc_1$ complex; Inhibitor; E-$\beta$-methoxyacyrlate; MOA-stilbene; Q-cycle; Iron–sulfur protein

## 1. Introduction

The cytochrome $bc_1$ complex forms the middle part of the respiratory chain in mitochondria and many aerobic bacteria. Its redox prosthetic groups are contained in three subunits, the diheme cytochrome *b*, cytochrome $c_1$, and the Rieske iron–sulfur protein [1]. The mitochondrial enzymes contain up to eight additional subunits which lack prosthetic groups [2].

The cytochrome $bc_1$ complex transfers electrons from ubihydroquinone to cytochrome *c* and links this electron transfer to the vectorial transport of protons across the inner mitochondrial or bacterial plasma membrane. The overall electron and proton pathways are described by the widely accepted protonmotive ubiquinone-cycle [1,3]. In this reaction scheme proton translocation occurs by oxidation of ubihydroquinone at center P (center o) with concomitant proton release on the positive side of the membrane; recycling of every second electron across the membrane to reduce

Abbreviations: Center N and P, ubiquinone reaction center on the negative and positive side of the membrane, respectively; MOA, E-$\beta$-methoxyacrylate

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*PII* S0005-2728(97)00060-1

ubiquinone at center N (center i) is linked to proton uptake from the negative side of the membrane.

Recent results by Ding et al. suggest that center P harbors two ubiquinone molecules [4]. This has prompted a discussion on possible mechanisms by which a concerted action of the two ubiquinones could ensure the bifurcated electron flow at this ubihydroquinone oxidation center. It has been proposed that this could be achieved in an edge-to-edge configuration of the headgroups by spatially separating the charges [5]. Alternatively, in a sandwiched configuration the reaction could be controlled electrostatically as suggested in the proton-gated charge transfer mechanism [6].

Inhibitor binding studies also suggest a rather complex situation at center P [7]. Removal of the 'Rieske' iron–sulfur protein significantly affects binding of hydroxynaphtoquinone-type inhibitors [8,9] and the $K_d$ of stigmatellin [10,11] increases dramatically. Quite in contrast, only a minor change in the affinity of E-$\beta$-methoxyacrylate inhibitors is observed. Still the redox-state of the iron–sulfur cluster has some influence on the $K_d$ of the E-$\beta$-methoxyacrylate inhibitors, changing it about 3 fold. In order to further characterize this interaction, we analyzed the pH-dependence of binding of the E-$\beta$-methoxyacrylate inhibitor MOA-stilbene [12] to isolated bovine cytochrome $bc_1$ complex.

## 2. Materials and methods

Bovine heart cytochrome $bc_1$ complex was prepared as described by Schägger et al. [13]. Chromatography on Sepharose CL-6B equilibrated with 0.05% Triton X-100, 100 mM NaCl, 2 mM $NaN_3$, 20 mM $Na^+$/Mops, pH 7.2 was the final step. Fractions containing cytochrome $bc_1$ complex were collected and concentrated to around 40 $\mu$M cytochrome *b* by ultrafiltration through a YM-100 membrane (Amicon). A preparation lacking the iron–sulfur protein and the smallest subunit of the cytochrome $bc_1$ complex was obtained in the presence of antimycin as described earlier [7,14]. Concentration of cytochrome $bc_1$ complex was determined as half of the heme *b* concentration, which was measured at 562–575 nm ($\epsilon = 28.5\ mM^{-1}\ cm^{-1}$).

Steady state activity of cytochrome $bc_1$ complex was recorded as the rate of cytochrome *c* reduction in a Shimadzu UV-300 spectrophotometer in dual-wavelength mode at 550–540 nm ($\epsilon = 19\ mM^{-1}\ cm^{-1}$) using a stirred cuvette with a final volume of 2 ml thermostatted at 25°C. Buffer: 50 mM KCl, 2 mM $NaN_3$, 0.2 mM EDTA, 50 mM $K^+$/Mops, pH 7.2. 0.1% bovine serum albumine was added to prevent rapid precipitation of the quinone. After addition of 50 $\mu$M horse heart cytochrome *c*, 75 $\mu$M nonylubihydroquinone was added. Then the reaction was started by the addition 2.5 nM cytochrome $bc_1$ complex. The slopes were corrected for the non-catalytic rate of cytochrome *c* reduction by hydroquinone observed prior to the addition of enzyme.

MOA-Stilbene [12] was dissolved in ethanol and its concentration was determined at 338 nm ($\epsilon = 26.5\ mM^{-1}\ cm^{-1}$). Apparent dissociation constants ($K_d$) for this inhibitor were determined at a concentration of 0.5 $\mu$M cytochrome $bc_1$ complex by direct fluorescence quench titration as described earlier [15]. The redox-state of the cytochromes was checked by recording a reduced *minus* oxidized spectrum after each titration. Buffer: 0.05% Triton X-100, 100 mM NaCl, 2 mM $NaN_3$, buffered with 50 mM $K^+$/Mes (pH 5.4–6.6), $K^+$/Mops (pH 6.8–7.8) or $K^+$/ Taps (pH 8.0–9.2). It should be noted that MOA-stilbene has no protonable group that would change its protonation state between pH 5.4 and 9.2. Also, Na-dithionite even at much higher concentrations than those used in the experiments reported here, does not modify or destabilize MOA-stilbene [12].

Data were analyzed using the Psiplot software package version 4.61 (Poly Software International). The numerical procedure used to fit experimental data was the Marquardt algorithm [16].

## 3. Results and discussion

The dissociation constant for MOA-stilbene binding to oxidized bovine cytochrome $bc_1$ complex at pH 7.0 was 24 nM as determined by fluorescence quench titration which is in accordance with the values obtained in earlier studies [7,15]. As titrations were performed at room temperature and took about 20 min, we tested whether the cytochrome $bc_1$ complex was sufficiently stable when incubated under these conditions at pH values between 5.0 and 9.5

(data not shown). A dramatic loss of activity was observed at pH 5.0 and 5.5, but upon incubation between pH 6.0 and 9.5, the activity assayed at pH 7.2 after 20 minutes was about 80% of the initial value. Some initial loss of activity was observed immediately following dilution of the enzyme into buffer at pH 8.0 and higher, but this was not followed by further loss of catalytic activity over time. Also between pH 6.0 and 9.5 no loss of heme *b* was observed in the reduced minus oxidized spectrum of the enzyme incubated at room temperature, which is known to be a sensitive indicator for the denaturation of this multiprotein complex (data not shown). We concluded that the isolated cytochrome $bc_1$ complex was stable enough to give reliable results when the fluorescence binding titrations were carried out between pH 6.0 and 9.5.

At pH 6.0 the $K_d$ for MOA-stilbene binding to oxidized cytochrome $bc_1$ complex was 2.5 times higher than at pH 9.5 (Fig. 1). The pH-profile could be fitted by assuming that a single protonable group was responsible for the change in affinity according to the following equation:

$$K_d = K_{d(A^-)} + \left\{1 + \frac{K_A}{[H^+]}\right\}^{-1} \times \left(K_{d(HA)} - K_{d(A^-)}\right), \quad (1)$$

where $K_d$ is the observed dissociation constant and $K_{d(HA)}$ and $K_{d(A^-)}$ the dissociation constants if a group with the acid-base equilibrium constant $K_A$ is protonated or deprotonated, respectively.

The parameters obtained for the curve shown in Fig. 1 were a $K_d$ of $27 \pm 1$ nM for the protonated and $8.7 \pm 0.6$ nM for the deprotonated form of a group with $pK_A = 7.7 \pm 0.1$.

The same $pK_A$ value has been reported earlier for one of the two redox-Bohr groups associated with the 'Rieske' iron–sulfur cluster [17–19]. Upon reduction, the $pK_A$ of this group shifts up several units to a value $> 10$. Evidence from circular dichroism-spectroscopy suggests that this group is one of the two histidines binding the iron–sulfur cluster [20].

To test whether the protonable group affecting MOA-stilbene binding was in fact identical to this redox-Bohr group associated with the iron–sulfur protein, we monitored pH-dependent binding of

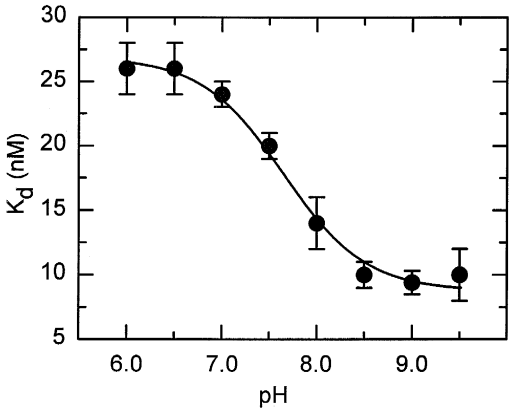


Fig. 1. pH dependence of MOA-stilbene binding to oxidized cytochrome $bc_1$ complex. The $K_d$ values for MOA-stilbene were determined at pH values between 6.0 and 9.5 as described in [15]. The error bars reflect the standard deviations from 10 individual titrations performed at each pH-value. The line represents the least squares fit using Eq. (1) given in the text. The fitted parameters were $pK_A = 7.7 \pm 0.1$, $K_{d(HA)} = 27 \pm 1$ nM and $K_{d(A^-)} = 8.7 \pm 0.6$ nM.

MOA-stilbene to cytochrome $bc_1$ complex in which the iron–sulfur cluster was reduced by ascorbate or in which the subunit carrying this redox-center was removed (Fig. 2). Over the entire pH-range the observed apparent $K_d$ was 3–4 times higher than the value obtained for the deprotonated oxidized complex. This is consistent with the redox-dependent binding of this inhibitor reported earlier [7,15].

For the preparation lacking the iron–sulfur protein (Fig. 2A), a constant $K_d$ of about 85 nM was found between pH 6.0 and 9.5. When the complete complex was partially reduced by adding 1 $\mu$M Na-ascorbate prior to the titration (Fig. 2B), a $K_d$ of about 100 nM was observed at lower pH but it dropped to 70 $\mu$M above pH 7.5. The addition of ascorbate leads to a complete reduction of the iron–sulfur center, but also reduces 50–100% of heme $b_H$ in a pH-dependent fashion (insert in Fig. 2B). Some influence of reduced cytochrome *b* on the binding of MOA-stilbene has been observed earlier for delipidated cytochrome $bc_1$ complex [7]. However, the increasing degree of reduction of cytochrome *b* did not go parallel with the observed change in $K_d$. Instead, the data for partially reduced enzyme again could be fitted using Eq. (1) but a somewhat higher $pK_A$ of 8.2 was obtained (see Fig. 2B). The effect of pH on the $K_d$ of

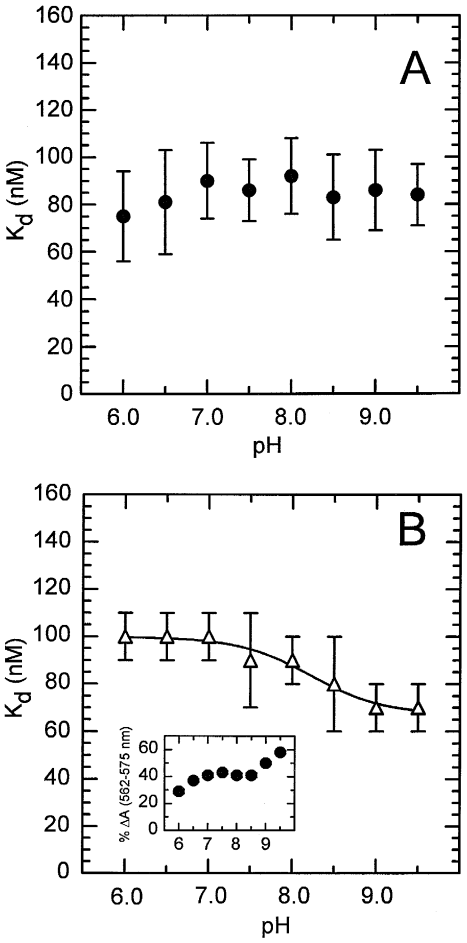


Fig. 2. pH dependence of MOA-stilbene binding to partially reduced and iron–sulfur-protein depleted cytochrome $bc_1$ complex. The $K_d$ values for MOA-stilbene were determined at pH values between 6.0 and 9.5 as described in [15]. The error bars reflect the standard deviations from $\geq 6$ individual titrations performed at each pH-value. (A) ●, iron–sulfur protein depleted complex. (B) △, complex partially reduced by adding 1 $\mu$M Na-ascorbate before starting the titrations. The line represents the least squares fit using Eq. (1) given in the text. The fitted parameters were $pK_A = 8.2 \pm 0.2$, $K_{d(HA)} = 100 \pm 2$ nM and $K_{d(A^-)} = 67 \pm 3$ nM. Insert: The percentage of the absorbance difference at 562–575 nm after addition of 1 $\mu$M Na-ascorbate under the same conditions as the binding assay as a measure for the fraction of reduced cytochrome *b* was plotted against pH. Due to the difference in the extinction coefficients between the to heme groups, 60% $\Delta$A at 562–575 nm means that 100% of heme $b_H$ but only very little heme $b_L$ was reduced.

MOA-stilbene binding to partially reduced cytochrome $bc_1$ complex is rather small, but the quality of the data seems to be sufficient to conclude that the change in $K_d$ was significant and that the $pK_A$ was different from the one observed for the oxidized enzyme. A redox-Bohr p$K$ of 8 has been found by Rich et al. from redox-titrations with submitochondrial particles for reduced heme $b_H$ in the presence of the center P inhibitor myxothiazol [21]. This would match the value obtained from fitting the binding data with ascorbate reduced complex. The midpoint potential of heme $b_H$ and the redox-Bohr pK associated with heme $b_H$ was reported to be slightly lower in the absence of inhibitor, which could also reflect some weak interaction between heme $b_H$ and the MOA-stilbene binding site.

Together with the complete disappearance of pH-dependence after removal of the iron–sulfur protein, the markedly smaller and shifted effect after reduction of the iron–sulfur cluster suggests that it is in fact the $pK_{ox} = 7.7$ redox-Bohr groups associated with this redox center which affects binding of MOA-stilbene to oxidized cytochrome $bc_1$ complex.

To further explore how the redox-state of the

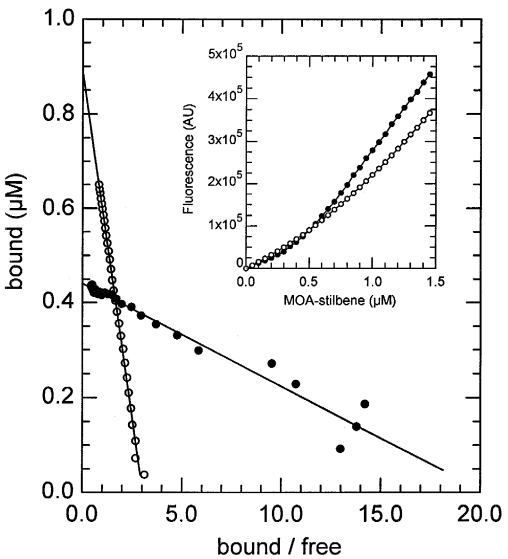


Fig. 3. MOA-stilbene binding to oxidized and fully reduced cytochrome $bc_1$ complex. Titrations with 0.5 $\mu$M cytochrome $bc_1$ complex at pH 7.0 were performed as described in [15]. A Scatchard representation reconstructed by using the parameters obtained by numerical analysis [15] of two typical binding titrations (insert) is shown to illustrate the difference in $K_d$ and the number of binding sites. (●) oxidized enzyme; (○) dithionite reduced enzyme.

heme-centers of cytochrome *b* influenced MOA-stilbene binding, we analyzed the binding of MOA-stilbene to cytochrome $bc_1$ complex which had been fully reduced with 2 $\mu$M Na-dithionite under anaerobic conditions. Under these conditions we observed a $K_d$ of $250 \pm 50$ nM which was independent of pH (not shown). This seemed to indicate that reduction of heme $b_L$ resulted in a further increase of $K_d$ and abolished or masked the minor pH effect observed in the partially reduced enzyme. However, interpretation of the dissociation constants obtained was difficult, as another quite unexpected observation was made: For fully reduced cytochrome $bc_1$ an additional binding site for MOA-stilbene was found consistently (Fig. 3). E.g., at pH 7.0, $1.8 \pm 0.1$ compared to $0.9 \pm 0.1$ binding sites for oxidized and partially reduced complex were found. We tried to use two independent $K_d$ values by modifying the equation used to fit the data [15], but numerical analysis was made impossible by the limited number of data points and the additional variable parameters required. By performing the binding titrations in the presence of 10 $\mu$M antimycin, which had no effect on the binding titrations, we excluded that the second binding site was center N (data not shown). On the other hand, both observed binding sites for MOA-stilbene were abolished by addition of 2 $\mu$M stigmatellin (data not shown). This can be taken as an indication that in reduced cytochrome $bc_1$ complex two molecules of MOA-stilbene can bind at center P, which seems not too far fetched as binding of two ubiquinones at this center has been reported [4]. This would still mean that only one of the two MOA-stilbene molecules bound to center P would induce the red-shift of the reduced cytochrome *b* spectrum [12,22,23], as binding titrations taking advantage of this spectral change show only one binding site [12].

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