Determination of the binding rate constants of stigmatellin and UHDBT to bovine cytochrome bc_1 complex by cytochrome c_1 oxidation

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Abstract Based on the high electron transfer rate between the [2Fe-2S] cluster and heme c_1 and the elevation of the redox midpoint potential of iron sulfur protein (ISP) upon binding of certain Qo inhibitors, the binding rate constants of stigmatellin and UHDBT to the cytochrome bc_1 complex were determined using a stopped-flow rapid scanning technique. Assuming that the intramolecular electron transfer from ISP to cytochrome c_1 is much faster than the binding of inhibitors, the rate of the inhibitor binding can be determined by the rate of cytochrome c_1 oxidation. The binding rate constants were calculated to be 1.0×10^5 and 2.3×10^5 M^{-1} s^{-1} at pH 7.5 for stigmatellin and UHDBT, respectively. The binding rate constant of UHDBT is pH dependent and that of stigmatellin is not.

Key words: Cytochrome bc_1 complex; Qo site inhibitor; Redox midpoint potential; Electron transfer; Binding rate

1. Introduction

Structural and functional studies of the complicated mitochondrial electron transfer systems require multiple approaches. In addition to conventional biochemical and biophysical studies [1–4], the use of specific inhibitors has greatly facilitated the establishment of electron transfer pathway [5–7]. Owing partially to the inhibitor studies, the proton motive Q-cycle mechanism has become accepted as the mechanism of electron transfer and proton translocation of the cytochrome bc_1 complex [8,9]. The inhibitors of the cytochrome bc_1 complex are classified into Qo and Qi inhibitors [10], depending on their site of action in the complex. The Qi site inhibitors, such as antimycin and 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide (NQNO), prevent binding of ubiquinone at the Qi site. The Qo site inhibitors, such as 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT), stigmatellin, myxothiazol and (*E*)-β-methoxyacrylate-stilbene (MOA-stilbene), block the binding of ubiquinol to the Qo site and thus inhibit electron transfer through this site.

On the basis of structural observations [11–14], the Qo site inhibitors are further divided into three categories: Qo-I, Qo-II and Qo-III [12]. Myxothiazol (Qo-I) does not change the redox midpoint potential (E_m) of the [2Fe-2S] cluster [5,6] whereas MOA-stilbene (Qo-I) slightly decreases the E_m. Stigmatellin (Qo-III) increases the E_m of the [2Fe-2S] cluster by 250 mV and UHDBT (Qo-II) increases the E_m by 70 mV [6].

The Qo-II and Qo-III inhibitors bind closer to the [2Fe-2S] cluster and Qo-I inhibitors bind closer to the heme bL. The binding of the Qo site inhibitors has considerable influence on the mobility of the head domain of iron sulfur protein (ISP). Binding of the Qo-I inhibitors increases the mobility of the head domain of ISP, as indicated by abolishing the anomalous signal for the [2Fe-2S] cluster in the electron density map [12]. In contrast to the Qo-I inhibitors, the Qo-II or Qo-III inhibitors cause a decrease of the mobility of the head domain of ISP.

Although biochemical and spectral studies of inhibitors have been extensive [10], the binding kinetics of the inhibitors have not been investigated. Taking advantage of the change of the E_m of ISP upon binding of the Qo-II and Qo-III inhibitors and the fast electron transfer between the [2Fe-2S] cluster and heme c_1, we have employed a stopped-flow rapid scanning technique [15,16] to determine the binding kinetics of the Qo-II and Qo-III inhibitors, stigmatellin and UHDBT, by measuring the rate of cytochrome c_1 oxidation. The rapid mixing methodology applied here allows monitoring of the oxidation or reduction of cytochrome c_1 with a time resolution of 1 ms after a dead time for mixing of 2 ms. Assuming that the binding kinetics of the inhibitors mimic substrate binding kinetics, the obtained data may be useful in the elucidation of the reaction mechanism of ubiquinol oxidation.

2. Materials and methods

2.1. Reagents and chemicals

Cytochrome *c*, type III, and sodium cholate were purchased from Sigma. Stigmatellin was purchased from Fluka. 2,3-Dimethoxyl-5-methyl-6-geranyl-1,4-benzoquinone (Q_2), and its reduced form (Q_2H_2) [17] and UHDBT [7] were synthesized as previously reported. Other chemicals were of the highest commercially available purity.

2.2. Bovine cytochrome bc_1 complex

The purified cytochrome bc_1 complex was prepared as previously reported [18]. It was dissolved in 50 mM Tris-HCl buffer, pH 7.8, containing 0.66 M sucrose to a protein concentration of 20 mg/ml and frozen at −80°C until use. The purified enzyme contains 10 nmol cytochrome *b* and 5.7 nmol cytochrome c_1 per mg protein. The complex as prepared is partially reduced with 60% of cytochrome c_1 and 83% of ISP in the reduced state.

2.3. Enzyme activity assay

The purified cytochrome bc_1 complex was dissolved in 50 mM K^+/Na^+ phosphate buffer, pH 7.4, containing 0.01% dodecyl maltoside and 1 mM EDTA to a protein concentration of 0.025 mg/ml. The assay mixture contains 50 mM K^+/Na^+ phosphate buffer, pH 7.4, 1 mM EDTA, 100 μM cytochrome *c*, and 25 μM Q_2H_2. The activity was determined by measuring the reduction of cytochrome *c* (the increase of absorbance at 550 nm) in a Shimadzu UV 2101 PC spectrophotometer at room temperature. A millimolar extinction coeffi-

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Abbreviations: ISP, iron sulfur protein; MOA-stilbene, (*E*)-β-methoxyacrylate-stilbene; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; E_m, redox midpoint potential

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cient of 18.5 was used to calculate the activity. Non-enzymatic oxidation of Q_2H_2, determined under the same conditions in the absence of enzyme, was subtracted.

2.4. Determination of the binding rate of the inhibitors

The cytochrome bc_1 complex was diluted in 20 mM Tris-HCl buffer, with various pHs (7.5, 8.3 and 9.2), containing 0.05% dodecyl maltoside and 0.1 M KCl to a cytochrome c_1 concentration of 7.5 μM and mixed with various concentrations (44–144 μM) of the inhibitors (stigmatellin or UHDBT) in the same buffer at room temperature in an Olis stopped-flow rapid scanning spectrophotometer. Oxidation of cytochrome c_1 was monitored by a decrease of absorption at 553.4 nm. A millimolar extinction coefficient of 17.5 was used in calculating the concentration of cytochrome c_1.

3. Results and discussion

3.1. pH or inhibitors induced electron transfer between the [2Fe-2S] cluster and heme c_1 in the native cytochrome bc_1 complex

The E_m of the [2Fe-2S] cluster is pH dependent and that of cytochrome c_1 is not. As pH decreases, the E_m of the [2Fe-2S] cluster increases [19]. At pH 7 the E_m of the ISP is 290 mV and that of cytochrome c_1 is 228 mV. At pH 8 the ISP and cytochrome c_1 have approximately the same E_m. When a cytochrome c_1 partially reduced cytochrome bc_1 complex at pH 8.0 was mixed with dilute acid to lower its pH to 7.0, oxidation of cytochrome c_1 was observed. On the other hand, when the pH of the enzyme solution was increased, cytochrome c_1 became more reduced, presumably at the expense of the oxidation of ISP. The rate of this acid/base induced electron transfer, from cytochrome c_1 to ISP and vice versa, is greater than what can be determined accurately by a conventional stopped-flow apparatus [20].

Upon the binding of stigmatellin to the cytochrome bc_1 complex, the E_m of the ISP is raised by 250 mV [6] whereas that of the cytochrome c_1 remains unchanged. The close binding sites of stigmatellin and UHDBT to the [2Fe-2S] cluster in the 3-dimensional structure provide a good explanation for the effect of these inhibitors on ISP (see Fig. 1). A similar but smaller E_m increase of the ISP was also reported for the binding of UHDBT. In the presence of a 2.5-fold molar excess of UHDBT the E_m of the ISP becomes 350 mV at neutral pH [21]. The shift of the E_m of the ISP thus causes electron transfer from cytochrome c_1 to ISP if cytochrome c_1 is in the partially reduced state. The degree of cytochrome c_1 oxidation can be simply assessed by the α-absorption of reduced cytochrome c_1 at 553 nm. The inhibitor induced reduction of ISP was also checked by a CD peak at 500 nm (data not shown). Myxothiazol binds closer to cytochrome b_L heme and causes

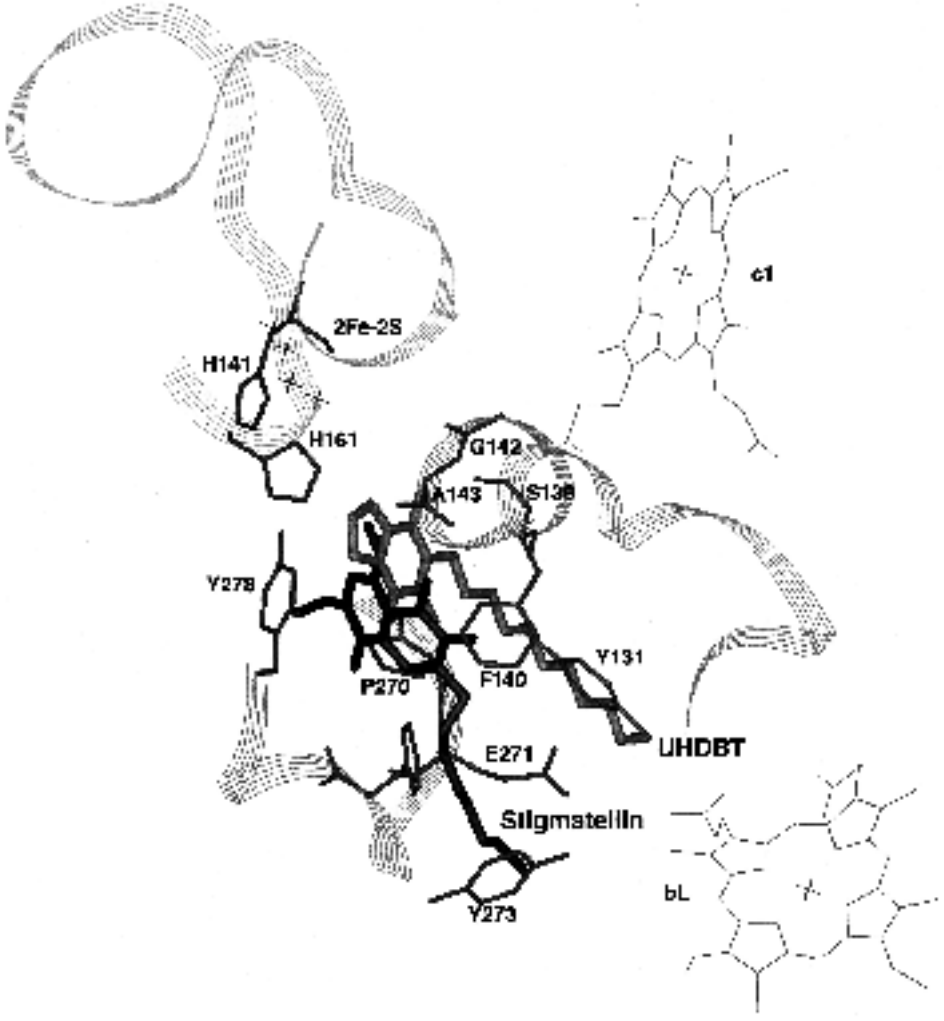

Fig. 1. Binding domains of stigmatellin and UHDBT in the Qo pocket of bovine cytochrome bc_1 complex.

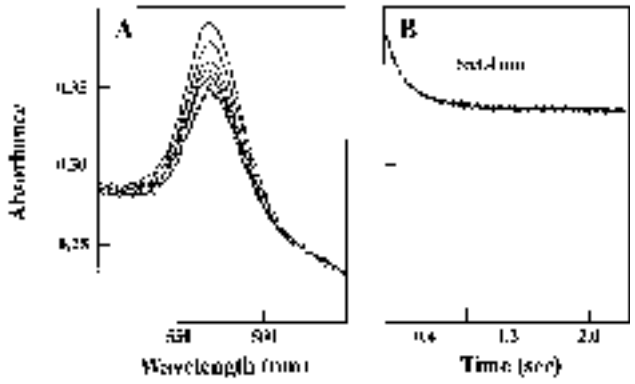

Fig. 2. Optical spectra and time trace obtained by stopped-flow rapid scanning spectrophotometry during the binding of stigmatellin at pH 8.3. Cytochrome bc_1 complex was diluted in 20 mM Tris-HCl, pH 8.3, containing 0.05% dodecyl maltoside and 100 mM KCl to a cytochrome c_1 concentration of 15 μM. The diluted complex was mixed with an equal volume of the buffer containing 88 μM of stigmatellin at room temperature in an Olis stopped-flow rapid scanning spectrophotometer. A: Representative traces showing the spectral change of oxidation of cytochrome c_1. B: The absorbance changes at 553.4 nm were taken from the data in A to monitor the rate of oxidation of heme c_1.

some spectral change, but has no effect on the E_m of the ISP [5]. MOA-stilbene slightly decreases the E_m of the ISP, and thus causes some reduction of cytochrome c_1.

Taking advantage of the high electron transfer rate between the ISP and cytochrome c_1 and the elevation of the E_m of ISP upon the binding of stigmatellin or UHDBT, we measured the rates of binding of these inhibitors to cytochrome bc_1 complex at various conditions by the conventional rapid mixing stopped-flow apparatus. It is assumed that pH and inhibitor induced electron transfers proceed with the same high rate and that inhibitor binding is the rate limiting step.

3.2. Determination of the binding rate constant of stigmatellin to the cytochrome bc_1 complex

Cytochrome c_1 partially reduced cytochrome bc_1 complex was diluted in 20 mM Tris-HCl buffer, pH 8.3, containing 0.05% dodecyl maltoside and 0.1 M KCl to a cytochrome c_1 concentration of around 15 μM. The diluted complex was mixed with an equal volume of the buffer containing stigmatellin at room temperature in an Olis stopped-flow rapid scanning spectrophotometer. The oxidation of cytochrome c_1 was monitored by the decrease of absorption at 553 nm (see Fig. 2). The binding rates of stigmatellin to the cytochrome bc_1 complex at pH 7.5, 8.3 and 9.2 were determined by following the rates of cytochrome c_1 oxidation. For each pH, the rates were measured using several stigmatellin concentrations (5-, 10-, 15-fold molar excess of the inhibitor to the cytochrome c_1). The plot indicates the rates of cytochrome c_1 oxidation are stigmatellin concentration dependent. From the rates of cytochrome c_1 oxidation at various concentrations of stigmatellin, the second order binding rate constant for this inhibitor at pH 7.5 was linearly fitted to be 1.0×10^5 M^{-1} s^{-1} (see Fig. 3). When the binding was determined at pH 8.3 and 9.2, the rate constants were not significantly different from that at pH 7.5.

This pH independent binding rate constant indicates either that no group with a pK_a between 7.5 and 9.2 is involved in the binding of stigmatellin to the cytochrome bc_1 complex or that the effect of a given group on the binding is compensated by the change of another group in the complex. A close contact between stigmatellin and the [2Fe-2S] cluster is expected because of the large increase of the E_m of ISP upon its binding. Structural analysis of cytochrome bc_1/inhibitor complex reveals that the electron density of stigmatellin is strongly connected to the [2Fe-2S] cluster at position H161 (Fig. 1). This may represent a hydrogen bond between (protonated) H161 ligand and a carbonyl and a methoxy group of stigmatellin or alternatively between deprotonated H161 and the hydroxy group of the inhibitor. The hydrogen bonding is responsible for holding the ISP in its fixed position [14].

3.3. Determination of the binding rate constant of UHDBT to cytochrome bc_1 complex:

Since binding of UHDBT also increases the E_m of ISP and has no effect on that of cytochrome c_1, binding kinetics of UHDBT can also be followed by the oxidation of cytochrome c_1. Similar binding experiments as those described for stigmatellin were carried out. The binding rates of UHDBT to the cytochrome bc_1 complex at three pHs were measured by the rates of cytochrome c_1 oxidation. For each pH, the rates were measured using several concentrations of UHDBT (5-, 10-, 17-, 23-fold molar excess of UHDBT to the cytochrome c_1). At pH 7.5 the rate constant for UHDBT binding was calculated to be $2.3\pm0.2\times10^5$ M^{-1} s^{-1} (see Fig. 4). In contrast to stigmatellin, the binding rate constant of UHDBT is pH dependent. A 10-fold decrease in binding rate constant was obtained when the binding was carried out at pH 9.2. At pH 8.3 the binding rate constant was $9.0\pm1.1\times10^4$ M^{-1} s^{-1}.

Although the pH dependence of the rate constant is most likely due to ionization of the hydroxyl group of UHDBT, a contribution of the protein is also important as pH dependent inhibition of a bromo-substituted inhibitor (UBrDBT) is also

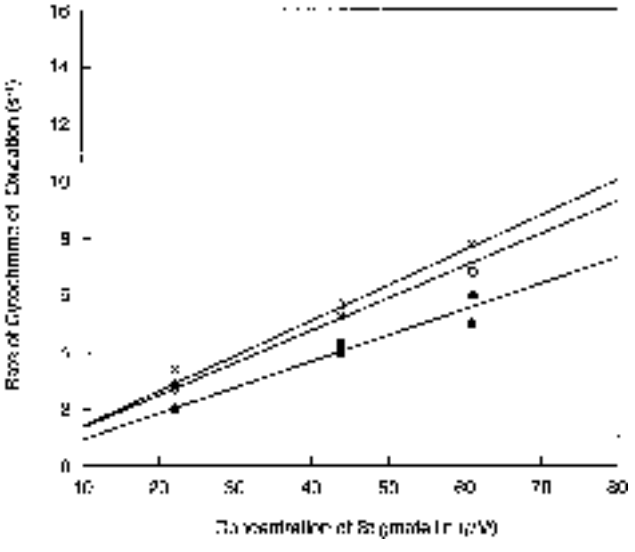

Fig. 3. The binding rates of stigmatellin to the cytochrome bc_1 complex at various pHs. The binding rates were measured by cytochrome c_1 oxidation at pH 7.5 (○), 8.3 (●) and 9.2 (×) in the presence of different concentrations of the inhibitor. The experimental condition is the same as that described in Fig. 2 except 7.5 μM of cytochrome c_1 and indicated amounts of stigmatellin were used. The plots of the slope of linear fit gave a second order rate constant of 1.0×10^5 M^{-1} s^{-1} for stigmatellin binding. The rate of binding is pH independent.

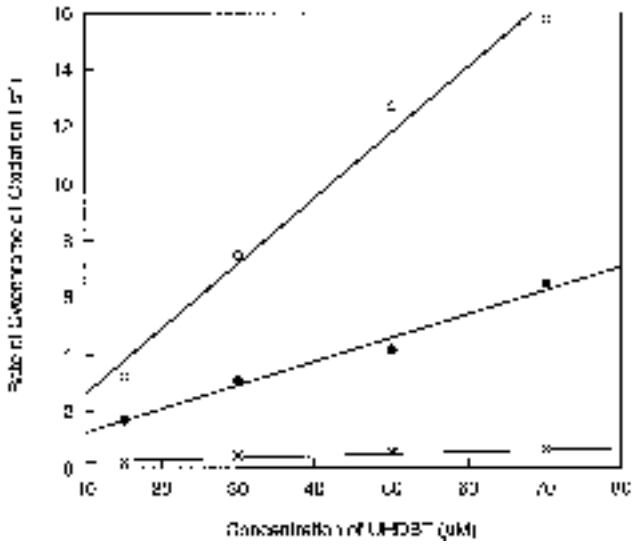

Fig. 4. The binding rates of UHDBT to the cytochrome bc_1 complex at various pHs. The experimental condition is the same as that described in Fig. 3 except for the presence of different concentrations of UHDBT. The binding of UHDBT is inhibitor concentration and pH dependent. The second order rate constants for UHDBT binding were $2.3\pm0.2\times10^5$ M^{-1} s^{-1} at pH 7.5 (○); $9.0\pm1.1\times10^4$ M^{-1} s^{-1} at pH 8.3 (●) and $2.3\pm0.1\times10^4$ M^{-1} s^{-1} at pH 9.2 (×).

observed [22]. The pH dependence correlated well with the inhibitory effect of UHDBT. The pK_a for the weak acid hydroxyl group of UHDBT was reported to be 6.5 [7].

From the structure of the cytochrome bc_1 complex, it is clear that quinone substrate or inhibitors reach their binding site through a common entrance, which is buried in or surrounded by phospholipid [11]. The amino acid residues surrounding the entrance and on the passage leading to the binding domains are generally hydrophobic. When pH is higher than the pK_a, UHDBT would be charged and unable to access the binding site. The binding site of UHDBT is on the edge of the Qo pocket in cytochrome *b* and close to the [2Fe-2S] center.

The anomalous scattering data indicate that in the fully oxidized cytochrome bc_1 complex less than 50% of the ISP is present in the fixed position [11], 31 Å from heme c_1, the rest are in the loose or released positions, somewhere between the 'fixed' (or '*b*') and the 'c_1' positions [13,14]. In the partially reduced cytochrome bc_1 complex the location of the [2Fe-2S] cluster is not yet firmly established. Preliminary results suggest that in the partially reduced complex the [2Fe-2S] cluster is located mostly at the loose or released positions because the electron density of the [2Fe-2S] cluster at the 'fixed' position is greatly diminished in the anomalous scattering map of the partially reduced cytochrome bc_1 complex. In the absence of UHDBT or stigmatellin, the pH induced electron transfer between the [2Fe-2S] cluster and heme c_1, either the acid induced cytochrome c_1 oxidation or the base induced cytochrome c_1 reduction [20], is very fast suggesting that ISP is constantly under rapid moving at the different released positions. The pH induced electron transfer becomes very slow if cytochrome bc_1 complex is treated with UHDBT or stigmatellin before the pH jump. This confirms that these inhibitors arrest the head domain of ISP to the fixed [11,12] or *b* position [14] regardless of whether the [2Fe-2S] cluster or heme c_1 is in the reduced or oxidized state. For kinetic analysis several events should be considered, including the inhibitor induced electron transfer from heme *c*1 to the [2Fe-2S] cluster, the interaction of the complex with the inhibitor when ISP is at the released positions and the movement of the ISP from the released positions to the fixed position in the inhibitor loaded complex. Apparently the electron transfer from heme c_1 to the [2Fe-2S] cluster in the cytochrome bc_1/inhibitor complex takes place before the [2Fe-2S] cluster moves to the fixed position. Therefore the observed rate constant of cytochrome c_1 oxidation is K_{on} for the binding of the inhibitor to the Qo pocket. Complex molecules with their ISP at fixed position are not able to accept electron from heme c_1 when they interact with inhibitor because of the long distance between the two redox centers. Therefore the measured rate constants may be lower than the actual ones, as the interaction of the complex molecules with ISP at fixed position is not accounted for.

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