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The non-equivalence of binding sites of coenzyme quinone and rotenone in mitochondrial NADH-CoQ reductase

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The fluorescent probe erythrosine 5'-iodoacetamide (ER) binds to mitochondrial NADH-CoQ reductase (Complex-I) accompanied by an enhancement of the fluorescence intensity. The binding of the CoQ analogue, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB), decreased the fluorescence intensity of the ER:Complex-I system. The 'site 1' inhibitor rotenone did not decrease the fluorescence intensity showing the non-identical nature of the binding sites of DB and rotenone. Also, the reduced form of DB did not decrease the fluorescence intensity. The decrease of the fluorescence intensity by DB was shown to be due to the removal of bound ER by DB. The rapid kinetics of ER binding was studied by temperature-jump relaxation. While DB caused complete elimination of the relaxation process in the ER:Complex-I system, rotenone caused only a decrease in the relaxation rate, suggesting conformational change. The relaxation rate showed a pH dependence with a maximum around pH 7.5.

NADH-CoQ reductase; Erythrosine-5'-iodoacetamide; Fluorescence probe; Temperature jump; Rotenone

1. INTRODUCTION

Mitochondrial NADH-CoQ reductase (Complex-I, EC 1.6.99.3) catalyzes the transfer of two electrons from NADH to ubiquinone-10 (CoQ) with a concomitant pumping of four protons across the membrane [1–3]. Rotenone is a classical inhibitor of the electron transfer to CoQ [4]. The significance of the rotenone binding site in the proton-pumping mechanism arises from the fact that only rotenone-sensitive electron transfer reactions drive the proton pump [4]. Electron transfer to none of the Fe-S centers of Complex-I is affected by rotenone [5,6]. Rotenone has been generally assumed to bind to the CoQ binding site but there is no hard evidence for this [7]. Recently the inhibition of Complex-I by pyridine derivatives has been implicated in idiopathic Parkinsonism [8,9]. The site of inhibition in this case and also by the recently discovered inhibitors such as capsaicin [10], arachidonic acid [11] and carbocyanine laser dyes [12] has been indicated as the rotenone/CoQ binding region. Inhibition by capsaicin has been correlated with the presence of an energy coupling site in various organisms [10]. Hence it has become important to map the inhibitor binding site with respect to that of CoQ. In addition, this region has been implicated as the most likely site of proton pumping [3]. Hence probing this region is expected to shed light on the mechanism of the proton pump also.

We have found that the fluorescent dye, erythrosine-5'-iodoacetamide (ER), binds to the CoQ binding region of Complex-I. This probe has been used to study the relationship of the CoQ binding site with that of rotenone. Our studies indicate that the two sites are not identical. This study has also revealed some interesting properties of this region.

Abbreviations: ER, erythrosine-5'-iodoacetamide; DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; PDB, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; T-jump, temperature jump; MES, 2-[*N*-morpholino]ethanesulphonic acid; MOPS, 3-[*N*-morpholino]-propanesulphonic acid; CAPSO, 3-[cyclohexylamino]-2-hydroxy-1-propanesulphonic acid; Tris, Tris(hydroxymethyl)aminomethane.

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2. MATERIALS AND METHODS

Complex-I [13] and DB [14] were prepared as described. ER was obtained from Molecular Probes (Eugene, OR, USA) and rotenone and NADH from Sigma Chemical Co.

Fluorescence measurements were carried out in a Shimadzu RF540 spectrofluorometer. Temperature-jump experiments were performed in a home-made instrument [15] interfaced to a home-made digital data aquisition system. The ultrafiltration experiments were carried out in an Amicon Ultrafiltration Cell fitted with a YM30 membrane. The samples were taken in 3 ml of buffer (250 mM sucrose, 10 mM MOPS, pH 7.5) and about 0.6 ml of the initial filtrates were assayed fluorimetrically (excitation at 525 nm and emission at 550 nm) for the concentration of ER. Other experimental details are given in the legends to figures.

3. RESULTS AND DISCUSSION

3.1. *Binding of ER to Complex-I*

Er binds to Complex-I as shown by (i) the enhance-

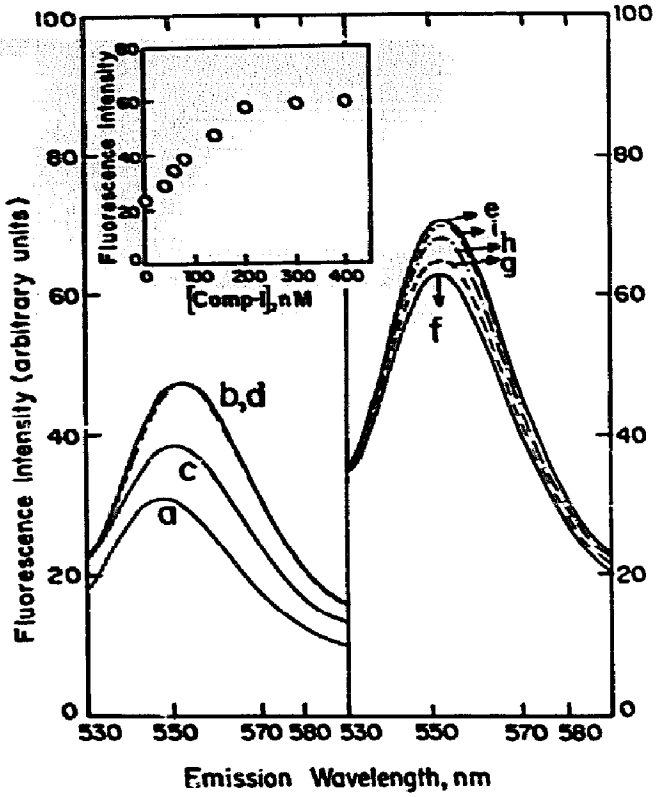

Fig. 1. Binding of ER to Complex-I and the effect of DB. Trace a shows the fluorescence emission spectrum of free ER (1.0 μM) in buffer (250 mM sucrose, 10 mM MOPS, pH 7.5). The excitation wavelength was 505 nm. Trace b shows the enhancement of fluorescence intensity upon addition of Complex-I (0.1 mg·ml^{-1}) to the sample a. Trace c shows the decrease in the fluorescence intensity of the ER:Complex-I due to the addition of DB (5 μM). Trace d shows the emission spectrum following the addition of rotenone (30 μM) to sample b. NADH reverses the action of DB. Trace e shows the emission spectrum of ER:Complex-I (1.5 μM of ER and 0.1 mg·ml^{-1} of Complex-I) and trace f shows the reduction caused by DB (1.0 μM). Traces g–i recorded at 1.0 min intervals after the addition of NADH (20 μM) to sample f show the reversal of DB-caused decrease in the fluorescence intensity. Complex-I titration of ER (inset). Fluorescence intensity of samples having 20 nM of ER and varying concentrations of Complex-I. The excitation was at 525 nm and emission at 550 nm. Complex-I concentrations were calculated by taking a value of 1 nmol·mg protein^{-1} [4].

ment of the fluorescence intensity of ER, and (ii) a red-shift of 5 nm in the emission spectrum of ER upon binding (Fig. 1). The titration of ER with Complex-I produced a hyperbolic curve (Fig. 1, inset) with saturation around 200–300 nM of Complex-I. A plot of $1/\Delta F$ vs. $1/E_0$ was linear (not shown) according to the standard equation $1/\Delta F = K_d/D_0E_0\Delta\Phi + 1/D_0\Delta\Phi$, where ΔF is the enhancement in fluorescence intensity. D_0 and E_0 are the total concentrations of ER and Complex-I respectively, and $\Delta\Phi$ is the change in fluorescence quantum yield. The dissociation constant K_d obtained was 0.15 μM assuming a 1:1 stoichiometry.

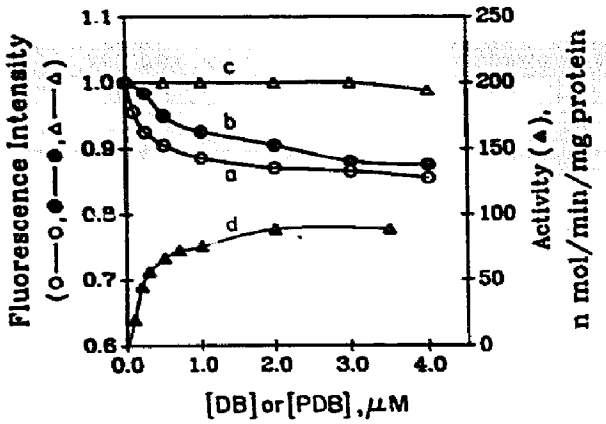

Fig. 2. Dependence of fluorescence intensity and the electron transfer activity on the concentration of DB. All the fluorescence intensity measurements (curves a–c) were done with samples having 0.05 mg·ml^{-1} Complex-I. Curves a and b show the dependences on the concentration of DB in the absence and in the presence of rotenone (40 μM) respectively. Curve c shows the dependence on the concentration of PDB. Curve d shows the dependence of NADH → DB electron transfer activity on the concentration of DB. The activity was assayed at 25°C, by following the fluorescence of NADH in samples having 0.06 mg·ml^{-1} Complex-I [6].

3.2. *CoQ analogue binding affects ER:Complex-I fluorescence intensity*

The CoQ analogue, DB has been widely used as a rotenone-sensitive electron acceptor of Complex-I [6,14]. Addition of DB to a suspension of ER and Complex-I caused a decrease in the fluorescence intensity (Fig. 1, trace c). This decrease showed a saturable dependence on the concentration of DB (Fig. 2). This was similar to the dependence of the rate of electron transfer from NADH to DB on the concentration of DB (Fig. 2). This suggests that the functionally relevant mode of binding of DB is responsible for the observed decrease in fluorescence intensity. Addition of DB to a suspension of either the detergent (cholate) or soybean lipid vesicles and ER did not cause any significant decrease in the fluorescence intensity. This shows that the binding site of ER in Complex-I is neither the contaminating detergent nor phospholipids present in Complex-I.

One of the following mechanisms may be responsible for the decrease of fluorescence intensity in the presence of DB. (*a*) DB binds close to the ER binding site such that the fluorescence of bound ER is partially quenched by the bound DB, and (*b*) DB competes for the binding site of ER. Addition of DB causes the removal of bound ER, hence the decrease in overall fluorescence intensity. In order to check whether the observed decrease in fluorescence intensity is due to displacement of bound DB, the aqueous concentration of ER was measured in ultrafiltration experiments. In a typical experiment, the presence of Complex-I (0.15 mg·ml^{-1}) in 0.17 μM ER solution reduced the concentration of ER in the filtrate to 0.10 μM. In the presence of Complex-I (0.15 mg·ml^{-1})

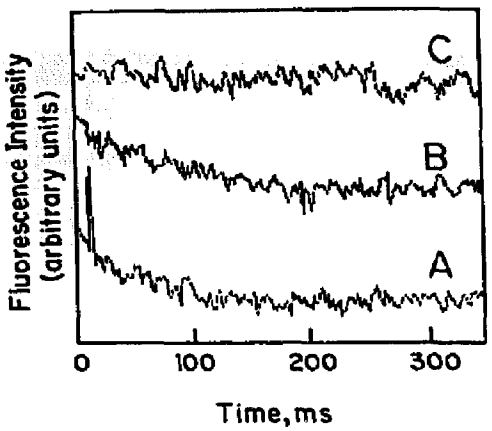

Fig. 3. T-jump traces of the ER:Complex-I with rotenone and DB. Trace A is a typical relaxation trace showing the decrease in fluorescence intensity of a solution of ER (1.67 μM) and Complex-I (0.37 μM) in buffer (250 mM sucrose, 25 mM KH_2PO_4, 100 mM KCl, pH 7.5) on giving a temperature jump of ~2.5°C at 25°C. Trace B shows the effect of adding rotenone (75 μM) to the above solution of ER and Complex-I. Trace C shows the elimination of the relaxation process in the presence of DB (2 μM). The origin of each trace is shifted vertically to avoid congestion. The exponential time constants of traces A and B are 44 ms and 66 ms, respectively. Each trace is an average of 7 experiments.

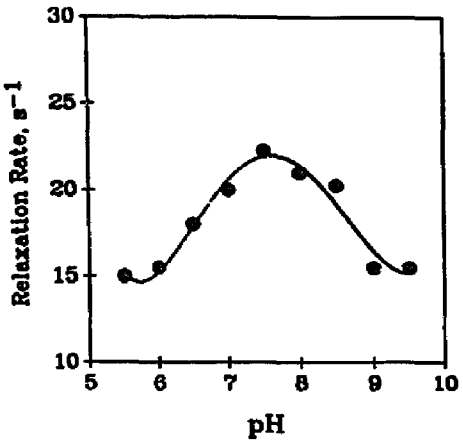

Fig. 5. pH dependence of ER binding kinetics. A mixed buffer containing 10 mM citrate, 10 mM MES, 10 mM Tris, 10 mM CAPSO, 250 mM sucrose and 100 mM KCl was adjusted to the desired pH to carry out the T-jump experiments. T-jump experiments were done with 0.37 μM of Complex-I and 1.67 μM of ER.

and DB (1.5 μM) the concentration of ER in the filtrate increased to 0.12 μM. These results clearly show that the free aqueous concentration of ER increased in the presence of DB. This supports the mechanism (*b*) mentioned above.

Addition of NADH relieved the decrease in fluorescence intensity caused by DB (Fig. 1, traces g–i). In view of the mechanism established above, this indicates that reduced DB does not cause the release of bound ER. It is likely that the binding sites of oxidised and reduced DB are not identical. The DB analogue PDB which lacks the decyl chain caused much less decrease of the fluorescence intensity (Fig. 2). Electron transfer to PDB is largely rotenone insensitive and hence its binding site is likely to be different.

The classical inhibitor rotenone did not cause any decrease in fluorescence intensity even at concentrations much higher than that required to inhibit electron transfer to DB (Fig. 1, trace d). (The electron transfer is inhibited more than 99% at about 1.5 μM rotenone.) This clearly shows that the binding sites of rotenone and DB are different. This conclusion is also supported by the observation that DB caused a decrease in fluorescence intensity even in the presence of rotenone (Fig. 2, curve b).

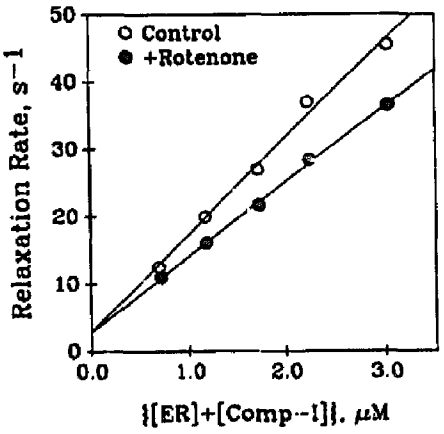

Fig. 4. The effect of rotenone on the DB binding kinetics. To a fixed concentration of Complex-I (0.37 μM) in buffer (as in Fig. 3), varying concentrations of ER were added and the temperature jumps were given. To each solution rotenone was added (75 μM) and temperature jumps were given to study the effect of rotenone on the relaxation process. {[ER] + [Complex-I]} was estimated by an iterative procedure [16]. The slope and the intercept give k_1 and k_{-1}, respectively. Uncertainties in τ measurements were less than 10%.

3.3. *Kinetics of ER binding*

The rapid kinetics of binding of ER to Complex-I was monitored by temperature-jump (T-jump) relaxation. A temperature jump of a suspension of Complex-I and ER resulted in a shift to a new equilibrium associated with a decrease in fluorescence intensity (Fig. 3). The timescale of this process was in the range of 20–100 ms. Fig. 4 shows the depencence of the relaxation rate, $1/\tau$, on the total concentration of Complex-I (E) and ER (D). Analysis of this dependence in terms of a single step process [16],

$$E + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ED$$

gave the rate constants k_1 and k_{-1} as 14.4 $M^{-1}s^{-1}$ and 2.90 s^{-1}, respectively. The dissociation constant K_d (= k_{-1}/k_1) calculated from the rate constants, 0.20 μM, agrees with the estimate obtained from Fig. 1, (inset).

Addition of DB caused a total elimination of the relaxation process (Fig. 3, trace C). This shows that the observed relaxation process (in the absence of DB) represents the binding of ER to the DB binding site alone. In contrast to DB, rotenone did not eliminate the relaxation process (Fig. 3, trace B) confirming once again the non-identical nature of the binding sites of DB and rotenone. However rotenone caused an increase in the relaxation time (Figs. 3 and 4). Analysis of concentration dependence of τ (Fig. 4) showed a significant decrease in k_1 (11.1 $M^{-1} \cdot s^{-1}$) with k_{-1} (2.93 s^{-1}) largely unchanged. The decrease in k_1 could be due to hindrance offered by the bound rotenone. In this picture, the rotenone binding site is not far from ER (and hence DB) binding site. Alternately, rotenone binding could have altered the conformation resulting in the decrease in k_1. Conformation changes induced by rotenone binding have been shown by cross-linking studies [7].

The rate of relaxation associated with the ER binding process showed a dependence on pH with a maximum around pH (Fig. 5). pH-dependent conformational change around the CoQ binding region could be the cause of this pH dependence. Since pH-dependent conformational change is a likely manifestation of a proton pump, the observed pH dependence (Fig. 5) is likely to have been caused by side chains involved in the proton pump of NADH-CoQ reductase. Further characterization of the ER binding region is currently under investigation using time-resolved fluorescence techniques.

REFERENCES

[1] Hatefi, Y. (1985) Annu. Rev. Biochem. 54, 1015–1069.
[2] Ragan, C.I. (1987) Curr. Top. Bioener. 15, 1–36.
[3] Weiss, H., Freidrich, T., Hofhause, G. and Preis, D. (1991) Eur. J. Biochem. 197, 563–576.
[4] Ragan, C.I. (1976) Biochim. Biophys. Acta 456, 249–290.
[5] Ohnishi, T. (1979) Membrane Proteins in Energy Transduction (R.A. Capaldi, Ed.), Marcel Dekker, New York, pp. 1–87.
[6] Krishnamoorthy, G. and Hinkle, P.C. (1988) J. Biol. Chem. 263, 17566–17575.
[7] Gondal, J.A. and Anderson, W.M. (1985) J. Biol. Chem. 260, 12690–12694.
[8] Ramsay, R.R., Salach, J.I., Dadgar, J. and Singer, T.P. (1986) Biochem. Biophys. Res. Commun. 135, 269–275.
[9] Sayre, L.M. (1989) Toxicol. Lett. 48, 121–149.
[10] Yagi, Y. (1990) Arch. Biochem. Biophys. 281, 305–311.
[11] Morri, H., Takeuchi, Y. and Watanabe, Y. (1991) Biochem. Biophys. Res. Commun. 178, 1120–1126.
[12] Anderson, W.M., Chambers, B.B., Wood, J.M. and Benninger, L. (1991) Biochem. Pharmacol. 41, 677–684.
[13] Hatefi, Y. and Rieske, J.S. (1967) Methods Enzymol. 10, 225–239.
[14] Wan, Y.P., Williams, R.H., Folkers, K.H. and Racker, E. (1975) Biochem. Biophys. Res. Commun. 63, 11–15.
[15] Prabhananda, B.S. (1977) Proceedings of the Symposium on Fast Reactions, Department of Atomic Energy, India, p. 317.
[16] Bernasconi, C.F. (1976) Relaxation Kinetics, Academic Press, New York.