The chromone inhibitor stigmatellin – binding to the ubiquinol oxidation center at the C-side of the mitochondrial membrane

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Stigmatellin, a chromone inhibitor acting at the Q_0 center of the bc_1 complex, binds to the heme b-566 domain of cytochrome b as well as to the Fe_2S_2 protein Its binding induces a shift of the α-band of heme b-566 to 568 nm It does not influence the ligand field of the heme b-562 center Concomitant with the red shift, stigmatellin gives rise to an alteration of the EPR line shape of the Fe_2S_2 cluster, namely linewidth narrowing and g value shifts at all 3 principal values The midpoint redox potential of the Fe_2S_2 protein is shifted from 290 to 540 mV

Cytochrome bc_2 *complex* *Inhibitor* *Fe_2S_2 protein* *EPR* *Cytochrome* b *Stigmatellin*

1. INTRODUCTION

A new antibiotic, stigmatellin, has been isolated from *Stigmatella aurantiaca* [1]. Its chemical structure has been determined [1,2] The molecule is composed of a chromone ring, substituted by a hydroxy, a methyl and two methoxy, and linked to an alkenyl side chain substituted by two methoxy and 3 methyl groups. The binding and inhibitory effects of stigmatellin have been shown to be different from antimycin, but closely resemble those of myxothiazol [3]. Both stigmatellin and myxothiazol [4,5] bind very tightly to the complex (apparent $K_d < 10^{-10}$ M) with a stoichiometry of one per cytochrome (cyt.) bc_1 complex. These investigators demonstrated the competition between stigmatellin and myxothiazol for the same binding site; the red shift of the cyt. *b*-566 spectrum normally induced by stigmatellin in beef heart submitochondrial particles could not be obtained after pretreatment of the particles with an excess of myxothiazol, and vice versa, a myxothiazol-induced cyt. *b*-566 red shift could not be obtained after pretreatment with stigmatellin. However, a difference in the precise mode of action of stigmatellin and myxothiazol was revealed by the following two lines of observation: (i) several mutant strains of *Saccharomyces cerevisiae* resistant to myxothiazol [4] exhibited no cross resistance to stigmatellin [3]; (ii) cyt. *b* reduction behavior in the presence of stigmatellin plus antimycin was different [3] from that in the presence of myxothiazol plus antimycin [5].

Here we report striking effects of stigmatellin on the EPR spectrum and the midpoint redox potential of the Rieske iron-sulfur cluster concomitant with its effect on the cyt *b*-566 optical spectrum. Both stigmatellin and myxothiazol react with the ubiquinol oxidation site of the cyt. bc_1 complex [6], but these two inhibitors greatly differ in the mode of binding to the site. The experiments were performed with isolated beef heart cyt. bc_1 complex [7] which gives much higher optical resolution than in submitochondrial particles.

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2. RESULTS

2.1. *Effect of stigmatellin on the cyt.* b-*566 spectrum*

Stigmatellin induces a red shift of the cyt *b*-566 signal of cyt. *b* (fig.1, solid lines) The maximum lies at 568 nm, the minimum at 557 nm; the spectrum has a shoulder at 562 nm which may arise from the two overlapped red shift spectra of cyt. *b*-566 and *b*-558 by stigmatellin For comparison, the red shift spectrum induced by the binding of antimycin, a 'Q_i' site inhibitor, is also shown (fig.1A, dotted line) The maximum of this spectrum – a shift spectrum of the cyt. *b*-562 center of cyt *b* – lies at 564 nm; the minimum at 557 nm. The proportion of the two shift spectra is about 3:2, analogous to that of the two α-absorbance signals of cyt. *b*-562 and *b*-566. Fig.1B compares the stigmatellin-induced shift spectrum with that induced by myxothiazol, a 'Q_o' site-group I inhibitor [8] (fig.1B, dashed line) The myxothiazol spectrum has its maximum at 567 nm, the minimum being at 557 nm. It is somewhat smaller than the stigmatellin shift spectrum, since the myxothiazol red shift amounts to only 1 nm instead of 2 nm.

Fig.2 compares the shift spectrum obtained when stigmatellin and antimycin were added together (the sequence of addition plays no role) with that of the sum of the individual shift spectra, which was obtained by using a computer memory system. The two spectra are nearly superimposable which shows that the shift phenomena are additive, indicating that the binding of the two classes of inhibitors occurs independently

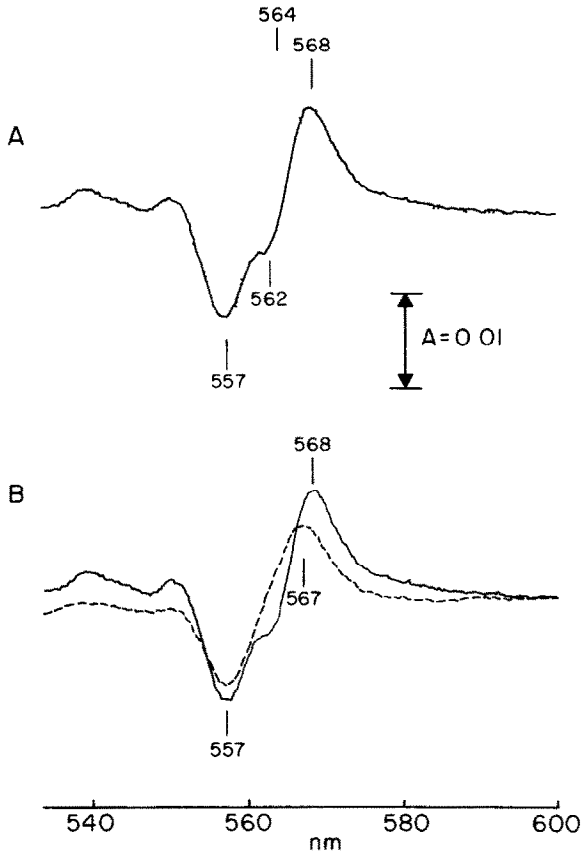

Fig 1 Red shift spectra of ferrocyt *b* induced by binding of stigmatellin, antimycin and myxothiazol (A) Red shift spectra of the cyt bc_1 complex induced by stigmatellin (——) and antimycin (···), (B) red shift spectra of the bc_1 complex induced by stigmatellin (——) and myxothiazol (– – –) The antibiotics were added at saturating levels 10 μM stigmatellin, 10 μM myxothiazol, and 10 μM antimycin, 3 μM (in cyt c_1) of the bc_1 complex which was dissolved in 0 1 M NaCl, 30 mM Mops, 0 1% Triton X-100, pH 7 2 The complex was reduced with dithionite

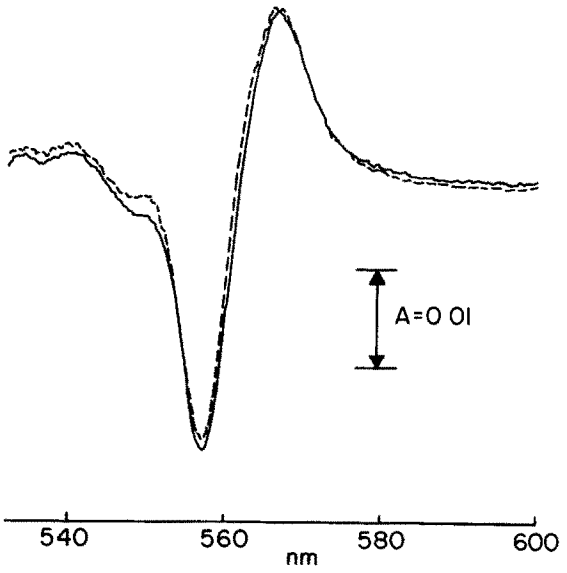

Fig 2 Comparison of the effect of adding stigmatellin and antimycin together (– – –) vs the sum of the effects after adding each inhibitor to a separate sample (——) Experimental conditions as in fig 1 The sum of the stored individual red shift spectra was obtained by adding the separate spectra by computer

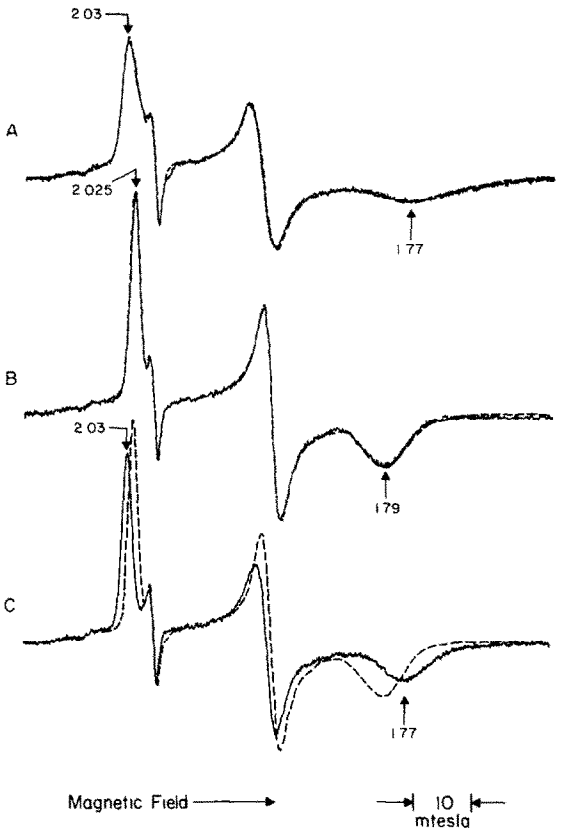

Fig 3 Effect of the Q_o site inhibitors, myxothiazol, stigmatellin and UHDBT on the EPR line shape of the Rieske iron-sulfur cluster and their competition for the same binding site Cyt bc_1 complex was dissolved in 0 1 M NaCl, 30 mM Mops (pH 7 2 and 0 1% Triton X-100 at final concentrations of 33 5 μM cyt c_1) The complex was reduced by 5 mM ascorbate All inhibitors added first were at the final concentration of 66 μM Cyt. bc_1 complex were treated with 3 inhibitors as follows: (A) (——) the complex was incubated for 3 min with myxothiazol alone, (– – –) incubated for 3 min with myxothiazol and then an additional 10 min with 330 μM stigmatellin, prior to the freezing of the sample (B) (——) Stigmatellin alone, (– – –) stigmatellin and then 660 μM myxothiazol in the same way as described above (C) (——) UHDBT alone, (– – –) UHDBT and then 66 μM stigmatellin EPR conditions microwave power, 1 mW, modulation amplitude, 1×10^{-3} T; time constant, 0 128 s, scanning rate, $2\,5 \times 10^{-2}$ T/min, sample temperature, 15 K All spectra were averaged 3 times

2.2 *Effects of stigmatellin on the Rieske iron-sulfur protein*

Concomitant with the induction of the red shift of the cyt. *b*-566 chromophore, stigmatellin gives rise to an alteration of the EPR line shape of the Rieske iron-sulfur cluster, namely, linewidth narrowing and *g* value shifts at all 3 principal values (fig.3B, solid line), which are somewhat more pronounced than those of UHDBT (fig.3C, solid line), a Q_o site-group II inhibitor [9,10] Myxothiazol does not induce a spectral broadening of the g_x signal in the cyt. bc_1 complex isolated with Triton X-100 [7] (fig.3A, solid line), in contrast to the observations obtained with succinate-cyt. C oxidoreductase [6] or cyt. bc_1 complex [11] isolated with cholate [12] This may result from a lack of ubiquinol bound to the Q_o site (0.1 mol UQ per mol bc_1 complex (M Deli Eposti, G. von Jagow and G. Lenaz, unpublished) as indicated by a broad g_x signal seen with the ascorbate reduced complex [13]. Stigmatellin competes with both myxothiazol and UHDBT for the same binding site. Stigmatellin has no effect on the EPR spectra

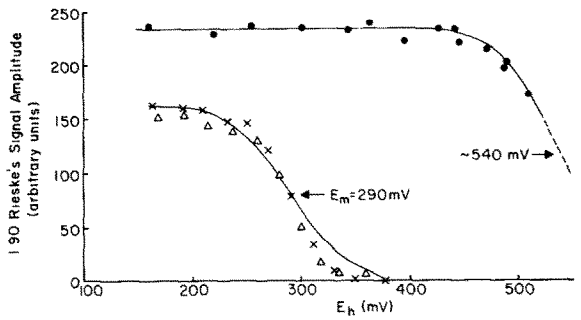

Fig 4 Effect of stigmatellin and myxothiazol on the midpoint redox potential of cyt bc_1 complex The complex at the final cyt c_1 concentration of 22 1 μM was titrated potentiometrically at room temperature according to [14] The final inhibitor concentration was 44 μM Redox mediators added were 40 μM DAD, 40 μM TMPD, 40 μM quinhydrone and 20 μM PMS. Dithionite was used as a reductant and $K_3[Fe(CN)_6]$ was used as an oxidant to poise the E_h of the complex EPR conditions microwave power, 5 mW, modulation amplitude, $1\,25 \times 10^{-3}$ T Other conditions as in fig 3 The titration in the E_h range higher than 450 mV was conducted with minimal volume of 10% H_2O_2 in the presence of 2 μM yeast cyt *c* peroxidase (×—×) Control, (Δ—Δ) plus myxothiazol, (●—●) plus stigmatellin

of the Rieske iron-sulfur cluster if the site is already occupied by myxothiazol (fig.3A, dashed line). Conversely, myxothiazol (5-times in excess of stigmatellin) does not replace the bound stigmatellin within 10 min of incubation (fig.3B, dashed line) Addition of stigmatellin after UHDBT converts the EPR line shape to that obtained with stigmatellin alone (fig 3C, dashed line) This shows that stigmatellin binds more tightly to the Rieske iron-sulfur protein than UHDBT, displacing the latter inhibitor from the site.

The midpoint redox potential of the iron-sulfur protein of the bc_1 complex titrates at 290 mV (fig.4), similar to that of the iron-sulfur protein in the succinate-cyt *c* oxidoreductase, or in the chromatophore [6,9,10]. The binding of stigmatellin results in a striking positive shift of the midpoint potential of the iron-sulfur protein by about 250 mV (fig.4) Myxothiazol does not change the midpoint potential of the iron-sulfur protein in either the isolated bc_1 complex (fig.4) or in the succinate-cyt. *c* oxidoreductase [6] Again, an addition of stigmatellin subsequent to the myxothiazol treatment does not shift the midpoint potential of the Rieske iron-sulfur cluster.

3. DISCUSSION

All of the inhibitors which are so far known to bind to the iron-sulfur protein of the bc_1 complex at the cytoplasmic side of the mitochondrial membrane, such as UHDBT [9,10], HMHQQ [15] or UHNQ [16], are composed of a benzoquinone ring substituted in position 6 by a hydroxy group and linked in position 1 to an alkyl side chain and in positions 3 and 4 to a heterocycle. These inhibitors have been classified as group II inhibitors of cyt bc_1 complex [8]. It is tempting to speculate that the aromatic hydroxy group of the hydroxyquinones forms a hydrogen bond with a basic amino acid residue of the iron-sulfur protein in the reaction cleft of the ubiquinol oxidation center In a similar fashion, the chromone ring of stigmatellin is composed of a chinoid ring system, substituted by a hydroxy group, but the latter is linked to the ring system at a different position. The tremendous change in the midpoint redox potential of the iron-sulfur protein points towards a binding of stigmatellin to the iron-sulfur protein Obviously, the inhibitor binds 4 orders of magnitude more tightly to the reduced form of the iron-sulfur protein than to the oxidized form. Smaller E_m shifts of the Rieske iron-sulfur protein by Q_o inhibitors have been previously reported, for example, 70 mV by UHDBT [9,10] and 45 mV by UHNQ [16].

The pronounced cyt. *b*-566 red shift is indicative of a concomitant binding of stigmatellin to the cyt. *b*-566 part of cyt. *b*. Binding to the cyt *b*-566 may be sustained by the two methoxy groups linked to the chromone ring because all group I inhibitors, which bind to the cyt. *b*-566, contain a similar structure, namely, 5-methoxyacrylate If these structure-function predictions are substantiated, the binding of stigmatellin further strengthens the notion that both the cyt *b*-566 domain of cyt. *b* and the iron-sulfur protein constitute the ubiquinol oxidation site as proposed by cyclic models of the electron transfer in the energy coupling Site II [17–19]. This work still does not exclude contribution of additional subunits of the bc_1 complex to this reaction center. As shown in fig.1, a group I inhibitor, myxothiazol, induces a spectral red shift of the cyt. *b*-566, but alters neither the EPR spectral line shape (fig.3) nor the midpoint redox potential of the Rieske iron-sulfur cluster (fig.4). Myxothiazol, however, eliminates the midpoint potential shift caused by the type II inhibitor, UHDBT [6] Thus, myxothiazol seems to affect the Rieske iron-sulfur domain of the Q_o site by a steric displacement of UHDBT from its site of action. These observations indicate that a functional Q_o binding site requires both the cyt. *b* and Rieske iron-sulfur domain to be in certain specific conformations or alteration in one domain is transferred to the other of the Q_o binding site These two possibilities are being examined using various stigmatellin derivatives which give rise to varying levels of inhibition on the NADH oxidation by beef heart submitochondrial particles [3].

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REFERENCES

[1] Hofle, G., Kunze, B., Zorzin, C and Reichenbach, H (1984) Liebigs Ann Chem. 39, 1883–1904

[2] Kunze, B , Kemmer, T., Hofle, G and Reichenbach, H. (1984) J Antibiot 37, 454–462

[3] Thierbach, G., Kunze, B., Reichenbach, H and Hofle, G. (1984) Biochim. Biophys. Acta 765, 227–235.

[4] Thierbach, G and Michaelis, G. (1982) Mol Gen. Genet. 186, 501–506.

[5] Von Jagow, G. and Engel, W D (1981) FEBS Lett 136, 19–24.

[6] Von Jagow, G , Ljungdahl, P.O , Graf, P , Ohnishi, T and Trumpower, B L (1984) J Biol Chem 259, 6318–6326.

[7] Engel, W.D , Schagger, H and Von Jagow, G (1983) Z. Physiol Chem 364, 1753–1763

[8] Von Jagow, G and Link, T A. (1985) Methods Enzymol , in press

[9] Bowyer, J R., Dutton, P.L., Prince, R C and Crofts, A R (1980) Biochim. Biophys Acta 592, 445–460.

[10] Bowyer, J R., Edwards, C A., Ohnishi, T and Trumpower, B.L (1982) J Biol Chem 257, 8321–8330.

[11] De Vries, S , Albracht, S P J , Berden, J , Marres, C.A.M and Slater, E.C. (1983) Biochim. Biophys. Acta 723, 91–103

[12] Hatefi, Y (1978) Methods Enzymol. 53, 35–40

[13] De Vries, S , Albracht, S P.J., Berden, J A and Slater, E C (1982) Biochim. Biophys Acta 681, 41–53

[14] Dutton, P L (1978) Methods Enzymol 54, 411–435.

[15] Zhu, Q.S , Berden, J A., De Vries, S , Folkers, K , Porter, T and Slater, E.C. (1982) Biochim Biophys Acta 682, 160–167.

[16] Matsuura, K., Bowyer, J.R , Ohnishi, T and Dutton, P L. (1983) J Biol. Chem. 258, 1571–1579

[17] Mitchell (1976) J Theor Biol 62, 327–367.

[18] Crofts, A R., Meinhardt, S W., Jones, K R and Snozzi, M (1983) Biochim Biophys Acta 723, 202–218.

[19] Wikstrom, M , Krab, K. and Saraste, M (1981) Annu Rev Biochem 50, 623–655.