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The inhibition of quinol oxidation by stigmatellin is similar in cytochrome bc_1 and b_6f complexes

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Stigmatellin is shown to raise the midpoint potential of the Rieske FeS-center of isolated cytochrome b_6f complex from 320 to about 460 mV. This increase in potential is accompanied by a modified EPR spectrum, i.e., a shift of the g_y line and a sharpening of the g_z peak. An equivalent effect on the mitochondrial cytochrome bc_1 complex has been observed earlier. Stigmatellin therefore inhibits both types of cytochrome complexes in the same way, by altering the FeS center to an unfavorable reductant for cytochrome f or c_1^+. The decreased reduction of cytochrome f by quinol in the presence of stigmatellin is accompanied by an increased transient reduction of cytochrome b_6. This can be explained by an increased formation of semiquinone during quinol oxidation by a stronger oxidizing FeS center.

The antibiotic stigmatellin was purified from the gliding bacterium *Stigmatella aurantiaca* [1] and its inhibitory action on the mitochochondrial bc_1 complex was characterized in detail [2–5]. It affects the mitochondrial cytochrome bc_1 complex at the quinol oxidation site (Q_0 site), shifting the α-peak of cytochrome b-566 to the red and raising the redox potential of the Rieske-FeS center [4,5]. Stigmatellin also inhibits photosynthetic electron flow in chloroplasts, but at two sites, Photosystem II and the cytochrome b_6f complex [6]. However, a lack of influence on the spectrum of cytochrome b_6 and the potential of the Rieske center has been reported [7], implying different inhibitory mechanisms in the mitochondrial and chloroplast system. In contrast, a shift of the α-peak of b_6 has been found recently [8]. In further agreement with the mitochondrial cytochrome bc_1 complex, we show here that stigmatellin also raises the redox potential of the Rieske-FeS center in the cytochrome b_6f complex. Stigmatellin induced changes in the EPR-spectrum of the Rieske-FeS center observed by us differ from the ones reported in Ref. 7.

Abbreviations: Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; UHDBT, 5-*N*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

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Stigmatellin was a generous gift of Prof. G. Höfle/GBF Braunschweig. For all experiments described below, stigmatellin was freshly dissolved in ethanol and solutions (on ice) were not kept for more than 4 h. The quality of the stigmatellin solution was checked by comparison of its UV-spectrum with published data [1]. The cytochrome b_6f complex was prepared from spinach chloroplasts by our standard procedure using MEGA-9 as detergent [9]. The sucrose density gradient was replaced by a second ammonium sulfate precipitation for the final purification step [10]. The precipitate was resuspended and diluted to the desired concentrations with 50 mM 4-morpholineethanesulfonic acid (Mes)/NaOH (pH 6.7), 50 mM NaCl, 5 mM KCl. Plastoquinol-3 was obtained as before [11].

Kinetic traces were taken on a modified Aminco-Chance DW2 spectrophotometer equipped with a stopped-flow apparatus, described elsewhere [11]. For EPR-experiments a Bruker ER 200D X-band spectrometer was used. Redox titrations were carried out according to Ref. 12, using the redox mediators 2,3,5,6-tetramethyl-*p*-phenylenediamine, *N*-methylphenazoniummethosulfate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine and ferricyanide (each at 50 μM concentration) in 50 mM 4-morpholinepropanesulfonic acid (Mops), (pH 7.0). Desired ambient potentials were achieved by adding ascorbate or ferricyanide. It seems noteworthy that addition of the inhibitor to the redox mixture slowed down redox equilibration considerably (several

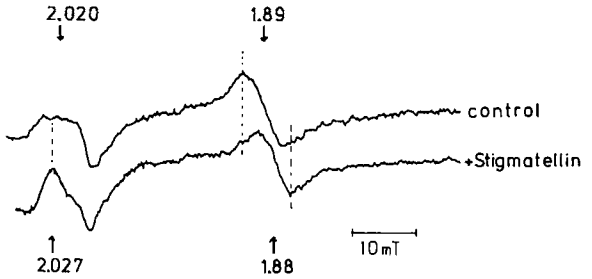

Fig. 1. EPR spectra of the Rieske-FeS center in the presence and absence of stigmatellin. Cytochrome b_6f complex was 5 μM in cytochrome f and stigmatellin was present at 20 μM (where indicated). The Rieske center was reduced by 5 mM ascorbate. EPR conditions: temperature, 15 K; microwave frequency, 9.44 GHz; microwave power, 6.3 mW; modulation amplitude, 1.6 mT.

minutes in the presence of stigmatellin). It could not be determined as yet if this is reflecting slow inhibitor binding or slow mediation.

Stigmatellin inhibited the plastoquinol-plastocyanin oxidoreductase activity of the isolated cytochrome b_6f complex, as reported before, 1 μM inhibiting about 50% with 50 nM cytochrome b_6f complex in the assay [6]. The inhibitor shifted the g_y line in the EPR-spectrum of the Rieske-FeS center from $g = 1.89$ to $g = 1.88$ and sharpened the broad g_z peak (Fig. 1). However, the sharpening and increase of the g_y line (without a shift) as reported in Ref. 7 was not observed. Redox titration of the g_y line yielded a midpoint at about 460 mV for the Rieske-FeS center in presence of stigmatellin, and at about 320 mV in its absence (Fig. 2). The value in absence of the inhibitor is higher than reported before [7,13], but comes close to 310 mV published for the isolated complex earlier [14]. It is possible that the stigmatellin-induced change of the midpoint potential of the Rieske-FeS center to an unfavorable, more positive value is responsible for the inhibitory action of

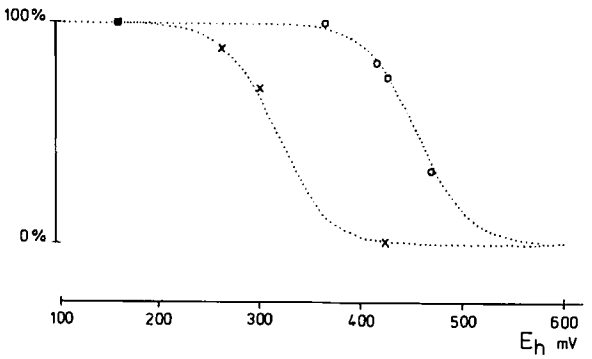

Fig. 2. Effect of stigmatellin on the redox titration of the g_y line in the EPR-spectrum of the Rieske-FeS center. The redox titration was done with purified cytochrome b_6f complex, 5 μM in cytochrome f. EPR spectra were obtained as described in Fig. 1. Open circles and crosses represent the measurements in presence (100 μM) and absence of stigmatellin, respectively. Theoretical titration curves with midpoint potential of 320 and 460 mV are shown in dots.

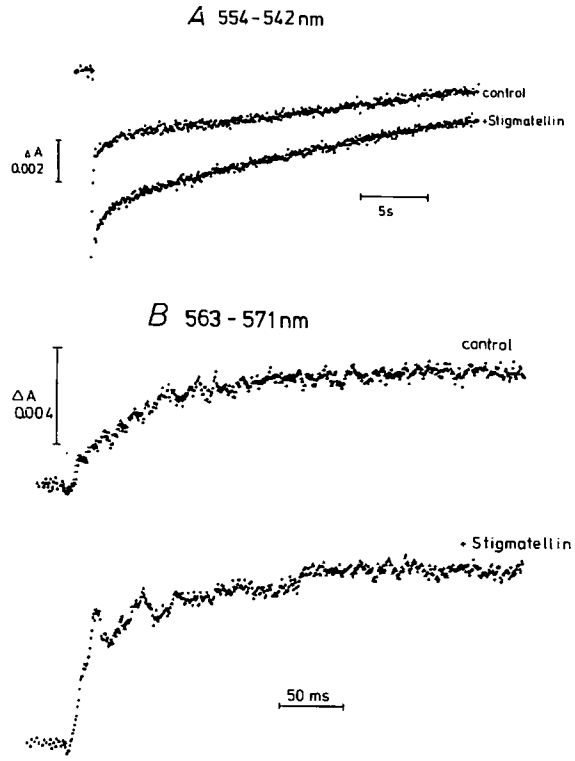

Fig. 3. Effects of stigmatellin on the fast reduction of cytochromes b_6 and f by plastoquinol. Fully oxidized cytochrome b_6 complex (final concentration, 0.5 μM) was reduced by plastoquinol-3 (final concentration, 20 μM) by stopped flow as described [11]. Where indicated stigmatellin (final concentration, 20 μM) was added to the syringe containing the complex. The traces represent averages of four measurements. Traces in A and B represent the reactions of cytochromes f and b_6, respectively.

stigmatellin on electron transfer from the Rieske center to cytochrome f. Indeed, it can be shown by EPR with rapidly frozen samples (less than 4 s) that in the presence of stigmatellin the Rieske-FeS-center is reduced by plastoquinol (measured by the appearance of the EPR line at $g = 1.89$), while cytochrome f stays largely oxidized (i.e., no loss of $g = 3.5$ signal occurred; data not presented).

The loss of rapid cytochrome f-reduction by plastoquinol in the presence of stigmatellin is documented in Fig. 3A, which shows an overlay of the kinetics in the absence and presence of the inhibitor. The residual slow reduction most probably represents the direct interaction of the quinol with the heme of cytochrome f. About 60% of cytochrome f is rapidly reduced in the control, and less than 20% in the presence of stigmatellin. At the same time the extent of fast reduction of cytochrome b_6 increases from 25 to about 37% of the full cytochrome b_6 complement in the presence of the inhibitor (Fig. 3B). This observation can be explained by the concerted reaction of plastoquinol with the Rieske-FeS center and low-potential cytochrome b_6 at the quinol oxidation site of the complex [15]. It is

thought that plastoquinol forms a semiquinone upon oxidation by the FeS center, and that this is the reductant of the low-potential heme of cytochrome b_6. Therefore the amount of semiquinone transiently formed may be increased by the more positive redox potential of the FeS-center, and this might lead to a larger transient reduction of cytochrome b_6.

These considerations are, of course, based on the assumption that the observed reduction of the *b*-cytochromes in the presence of stigmatellin does not reflect electron donation via the Q_R site. Published data (for a review see Ref. 16) as well as intensive examination of this question by the authors show that this is true throughout the physiological pH range and that this is most probably due to the very low (compared to bc_1 complexes) midpoint potentials of the two cytochrome *b* hemes (-50 mV and -170 mV).

In our experiments stigmatellin clearly affects both the midpoint potential and the EPR-spectrum of the Rieske-FeS center. In the chloroplast enzyme the increase of the midpoint potential by 140 mV to $+460$ mV is not as drastic as in the cytochrome bc_1 complex, where a potential of $+540$ mV in the presence of stigmatellin is observed [4,5]. In addition we observe a red-shift on the α-peak of cytochrome b_6 by stigmatellin, similar to the red-shift found for the mitochondrial cytochrome bc_1 complex [4]. This shift was also recently reported by Rich [8], although it was not observed previously by Malkin [7].

We cannot explain the discrepancy of our results to those reported earlier [7]. However, recently a series of stigmatellin derivatives which differ only in the structure of the side chain have been comprehensively examined [5]. Although their influences on spectral parameters of cytochrome *b* and the Rieske center, and even on the midpoint potential of the Rieske center, are quite heterogeneous, they nevertheless all inhibit oxidoreductase activity. In this context it seems noteworthy that stigmatellin is rather unstable in solution [1], even at 4°C, as evidenced by progressive alterations of its UV-spectrum. Thus inconsistent result could easily originate from the use of slightly modified compounds, which nevertheless may still be inhibitory.

The observations presented in this paper suggest that stigmatellin inhibits the cytochrome b_6f and the bc_1 complex [4] by the same mechanism. Interestingly, in the presence of the inhibitor a rapid reduction of cytochrome *b* by quinol still occurs. Unfortunately, due to the necessity of freezing the EPR samples, the reduction of the Rieske center cannot be observed with the same high time resolution. Thus a slowing down of the FeS center reduction by stigmatellin cannot be directly excluded by experiments. However, as cytochrome *b* can only be reduced by the Q_O site semiquinone, a preceeding, or at least concomitant, reduction of the FeS center must be inferred. The most obvious explanation for these results would be to assume that stigmatellin binds close to site of quinol oxidation but does not replace quinol, thereby still allowing for a reduction of the FeS center during the first turnover. This would imply that inhibition is entirely due to the raised midpoint potential. More complicated alternatives can nevertheless be proposed: the reduction kinetics of the terminal electron acceptor plastocyanine in the oxidoreductase assay [9] in the presence of nonsaturating concentrations of stigmatellin is slowed down but still monophasic (Ref. 6 and our results). This clearly shows that the binding of stigmatellin to its site of inhibition is characterized by high on/off rates. Therefore a first-turnover quinol oxidation might be possible despite the fact that the substrate and inhibitor binding sites are actually overlapping. The second turnover, however, will be hampered by a highly reduced Rieske center due to the altered redox equilibrium between the FeS center and cytochrome *f*. Moreover the raised potential of the FeS center reflects a highly preferential binding of the inhibitor to complexes with reduced FeS center. Thus it might even depend on the ambient redox potential whether competition or the redox effect plays the predominant inhibitory role.

The only other efficient inhibitor of both types of complexes is 5-*N*-undecyl-6-hydroxyl-4,7-dioxobenzothiazole, abbreviated UHDBT [16], which also raises the redox potential of the Rieske-FeS center in the cytochrome bc_1 complex [17]. It increases the distance between g_z and g_x in the EPR spectrum, reflecting higher rhombicity of the crystal field. Increased rhombicity in the presence of UHDBT has also been found for the FeS center of the b_6 complex [18], but an effect on the redox potential has not been reported so far. Interestingly, however, UHDBT does not shift the spectrum of cytochrome *b* [19].

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