Stigmatellin Affects Both Hemes of Cytochrome *b* in Cytochrome *b6f*/*bc1*-Complexes

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Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Cytochrome *bc1*-Complex, Cytochrome *b6f*-Complex, Stigmatellin, Heme Absorption, Cytochrome Redox Potentials

Stigmatellin causes spectral changes of both hemes of cytochrome *b* or *b6* in cytochrome *bc1*/*b6f*-complexes. It also affects the redox potentials of all three hemes, including cytochrome *c1* and *f*, in addition to the dramatic rise of the redox potential it exerts on the Rieske FeS-center. We conclude that stigmatellin changes the overall conformation of the complexes.

Introduction

The antibiotic stigmatellin was purified from the gliding bacterium *Stigmatella aurantiaca* [1] and its inhibition of the mitochondrial cytochrome *bc1*-complex was characterized in detail [2–4]. Stigmatellin was found to affect the mitochondrial cytochrome *bc1*-complex at the ubiquinol oxidation site (Q_o-site), shifting the α-peak of cytochrome *b* to the red and raising the redox potential of the Rieske FeS-center [4, 5]. Stigmatellin also inhibits photosynthetic electron flow in chloroplasts, where it affects photosystem II in addition to the cytochrome *b6f*-complex [6]. The inhibitory mechanism on the cytochrome *b6f*-complex, in contrast to an earlier report [7], has recently been shown to be similar to the one on the mitochondrial complex [8], *i.e.*, stigmatellin dramatically raises the redox potential of the Rieske FeS-center, so that arriving electrons do not pass on to cytochrome *f*. Also a red-shift of the α-peak of cytochrome *b6* has been reported [9]. In a more detailed study we demonstrate here that this spectral effect is of complex nature in both, in the chloroplast – as well as in the mitochondrial system, and not only a simple absorption shift of the low-potential heme of cytochrome *b*/*b6* at the Q_o-site. Together with the spectral changes, changes of the redox potentials of the hemes, including the ones of cytochrome *c1* and *f*, not only of the Rieske FeS-center, occur upon addition of stigmatellin in both systems. We conclude that stigmatellin, like antimycin [10], affects the overall conformation of these complexes.

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Materials and Methods

Stigmatellin was a generous gift of Dr. G. Höfle/GBF Braunschweig. It was always freshly dissolved in ethanol before use, and the concentration of the solutions was controlled by the UV spectrum [1]. The cytochrome *b6f*-complex was prepared from spinach or pea chloroplasts by our standard procedure using MEGA-9 (nonanoyl-N-methyl glucamide, OXYL/Bobingen, F.R.G.) as detergent [11]. The sucrose density gradient was replaced by a second ammonium sulfate precipitation for the final purification step [12]. The precipitate was resuspended and diluted to the desired concentrations with 50 mM 2-N-morpholino-ethanesulfonic acid (MES)/NaOH, pH 6.7, 50 mM NaCl, 5 mM KCl. The cytochrome *bc1*-complex was prepared by the method of Ljungdahl *et al.* [13].

Optical spectra were measured with a Kontron Uvikon 850 dual beam spectrophotometer. Redox titrations with 4–7 μM cytochrome *b6f*- or *bc1*-complex, suspended in 100 mM Hepes-NaOH, pH 7.0, in absence or presence of stigmatellin (80 μM) were carried out according to Dutton [14] as described before [15], in a home-made holder for stirring, cooling and gassing the anaerobic cuvette in the Kontron spectrophotometer. Ten recordings were averaged at each potential for obtaining the differences caused by stigmatellin. The spectra have been corrected for any dilutions during the titrations.

Results and Discussion

The overall spectral change of the α-peak region of the cytochrome *b6f*-complex caused by stigmatellin has been observed before [9]. It is shown again in Fig. 1, and at expanded scale in Fig. 5, top curve. The absolute spectra of the reduced complex indicate a slightly deeper valley between the absorptions of cytochromes *f* and *b6* in presence of stigmatellin (Fig. 1; a corresponding observation is made in Fig. 6 for the cytochrome *bc1*-complex). The change is not just a simple red-shift of one component, but more complex. A negative absorption at 559 nm, with a shoulder at 562.5 nm, and a positive peak at 567 nm is observed in Fig. 5. The complex nature of the change suggests that more than one heme group is affected.

The two hemes of cytochrome *b6* in the *b6f*-complex [16] have slightly different α-peak absorptions. At low *T* the high-potential heme shows a split α-peak, while the absorption of the low-potential heme is almost symmetrical [17, 18]. Corresponding absorption differences are seen at RT and have been exploited to follow individual redox kinetics of the two hemes in cytochrome *b6* [19]. The spectra at RT and the difference between them are shown again for the *b6f*-complex isolated from pea (Fig. 2). In contrast to a personal communication of P. Rich/Bodmin, they are identical to the ones for spinach (ref. [19] and Fig. 3 and 4). The high potential form shows a shoulder on the low wavelength side, while the low potential form is very slightly asymmetric to the high wavelength side.

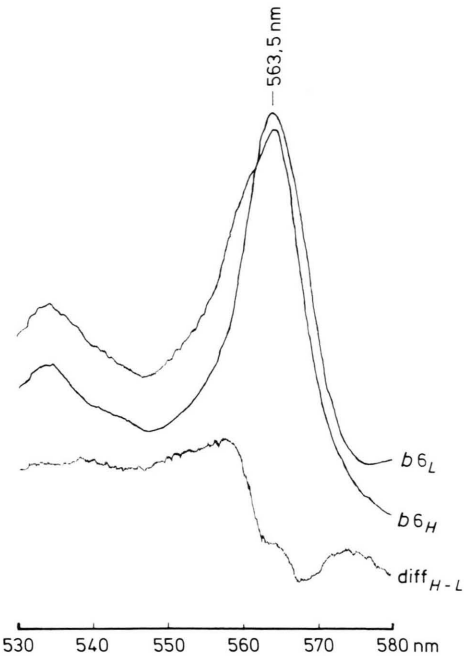

Fig. 2. Spectra of high- and low-potential cytochrome *b6* in the complex from pea. The spectrum for the low-potential form ($b6_L$) was obtained by subtraction of a spectrum at −180 mV ambient redox potential (cytochrome *b6* about 80% reduced) from the spectrum after full reduction (−390 mV). The spectrum for the high-potential form ($b6_H$) was obtained by subtracting a spectrum at +100 mV (only cytochrome *f* reduced) from a spectrum at −70 mV (about 20% of cytochrome *b6* reduced). The peaks have been normalized to the same height. The difference between the high- and low-potential form after normalization is also shown (diff H−L). The cytochrome complex was 7 μM in cytochrome *f* in the redox titration cuvette.

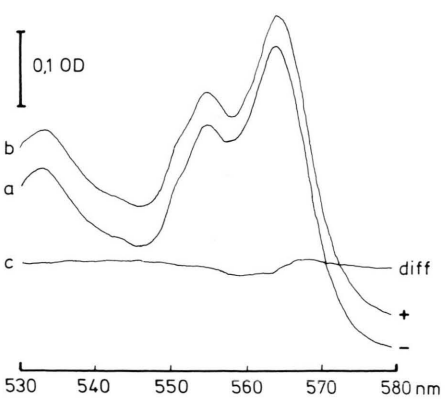

Fig. 1. Effect of stigmatellin on the spectrum of reduced cytochrome *b6f*-complex from spinach. The complex (7 μM in cytochrome *f*) was reduced by excess dithionite and its spectrum was measured as described under Methods, before (curve a, −) and after (curve b, +) addition of stigmatellin (80 μM final concentration). Curve c (diff) shows the difference caused by stigmatellin.

Fig. 3 and 4 show the effect of stigmatellin on the spectra of the low- and the high-potential heme of spinach cytochrome *b6*, respectively, which are included also in Fig. 5 at expanded scale. Unexpectedly, the larger change is found for the high-potential heme, which is the heme distal to the quinol-oxidation site where stigmatellin is thought to bind [4, 8]. The two changes partially compensate each other. The effect on the high-potential form is a red-shift which results from a less asymmetric α-peak in the presence of stigmatellin (Fig. 4). The effect on the

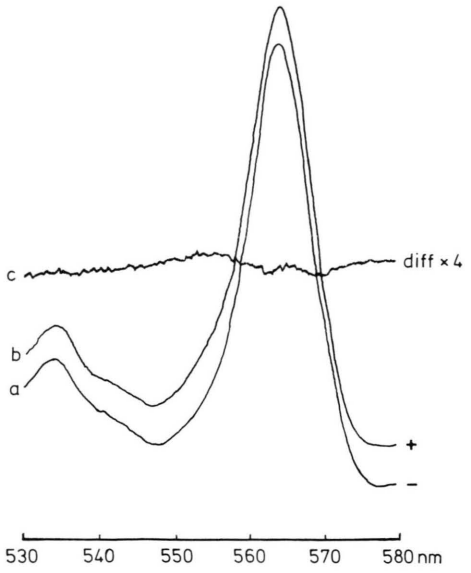

Fig. 3. Effect of stigmatellin on the spectrum of low-potential cytochrome *b*6 from spinach. The spectra for low-potential cytochrome *b*6 in presence (curve b, +) and absence (curve a, −) of stigmatellin were obtained as described for Fig. 2. The peaks have been normalized to the same height. The effect caused by addition of stigmatellin is shown at 4-times expanded scale (diff ×4).

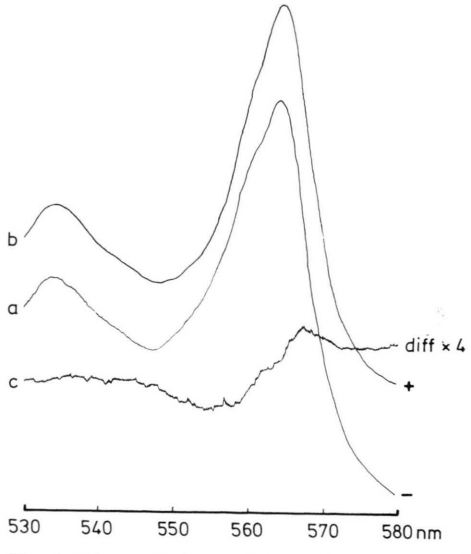

Fig. 4. Effect of stigmatellin on the spectrum of high-potential cytochrome *b*6 from spinach. The spectra for high-potential cytochrome *b*6 in presence (curve b, +) and absence (curve a, −) of stigmatellin were obtained as described for Fig. 2. The peaks have been normalized to the same height. The effect caused by addition of stigmatellin is shown at 4-times expanded scale (diff ×4).

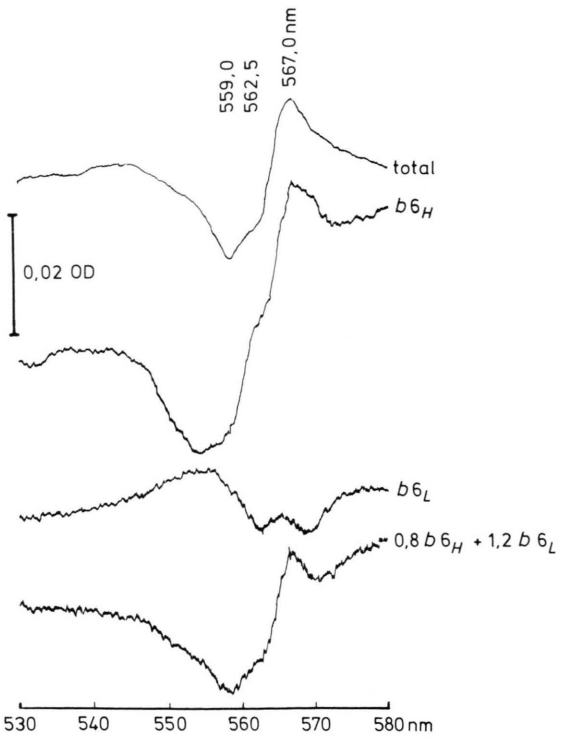

Fig. 5. Effects of stigmatellin on cytochrome *b*6 at expanded scale. The top spectrum shows the total change, and corresponds to trace c in Fig. 1, expanded 8-fold. Characteristic wavelengths of this change are indicated on top. The curve denoted $b6_H$ corresponds to the effect of stigmatellin expected for total high-potential cytochrome *b*6 (the change observed was normalized to half of total cytochrome *b*6 observable). The curve denoted $b6_L$ shows the same for total low-potential cytochrome *b*6. The bottom curve depicts the sum of 0.8 $b6_H$ plus 1.2 $b6_L$.

low-potential form corresponds to a narrowing of the α-peak (Fig. 3), possibly reflecting a loss of oscillatory modes in a stiffer environment. The overall effect of stigmatellin on the α-band absorption of cytochrome *b*6 (Fig. 5, top curve) can best be resynthesized (Fig. 5, bottom curve) by 80% of the effect on the high-, plus 120% of the effect on the low-potential heme. This resembles the contributions of the two forms to the total height of the α-peak during redox titration [15], and demonstrate that the extinction coefficient for the α-peak of the low-potential form is about 1.5 times the one of the high-potential form. Taking a ε_{mM} of 21 cm^{-1} at 563 nm for total heme in cytochrome *b*6 [20], the ε_{mM} for the high- and the low-potential heme are 16.8 and 25.2 cm^{-1}, respectively.

In mitochondria it has been found that stigmatellin and antimycin cause two independent red-shifts of the α-peak of cytochrome *b* [4], in accordance with the view that stigmatellin binds to the quinol-oxidation site and antimycin to the quinone-reduction site [10, 16]. Consequently it has been concluded, that antimycin shifts the absorption of the high-potential heme, while stigmatellin affects the low-potential heme of cytochrome *b* in the *bc1*-complex of mitochondria [4]. In contrast, the major effect of stigmatellin on cytochrome *b*6 is found for the high-potential form, as documented above. Since the inhibitory action of stigmatellin seems to be similar in the *b*6*f*- and the *bc1*-complex [8], we investigated this contradiction by taking a closer look to effects on individual hemes also in the mitochondrial complex.

Fig. 6 and Fig. 9 (top curve) present the overall change of the α-peak absorption of isolated mitochondrial cytochrome *bc1*-complex again [4]. Like the change of the *b*6*f*-complex it is not a simple red-shift. It shows negative peaks at 557.3 and 564.2 nm, and positive peaks at 561.7 and 568.3 nm (Fig. 9, top), and again it is composed of contributions from both, the high- and the low-potential heme of cytochrome *b* (Fig. 7–9). In the mitochondrial case the absorption change of the high-potential heme looks like a narrowing of the α-peak (Fig. 8 and 9), while the low-potential heme shifts to the red, which reflects a slight decrease in α-peak splitting (Fig. 7 and 9; see ref. [21] for a review).

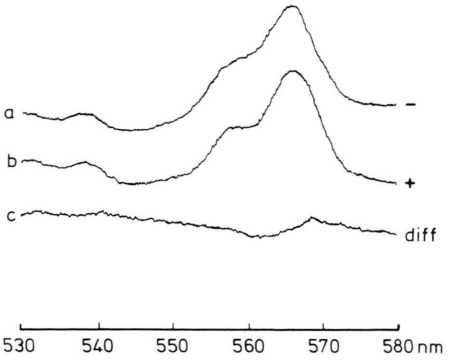

Fig. 7. Effect of stigmatellin on the spectrum of low-potential cytochrome *b* from beef heart. The spectra for low-potential cytochrome *b* in presence (curve b, +) and absence (curve a, −) of stigmatellin were obtained as the differences of fully reduced spectra (−220 mV ambient redox potential) minus the spectra recorded at −80 mV. The peaks have been normalized to the same height. The effect caused by addition of stigmatellin is shown in curve c (diff).

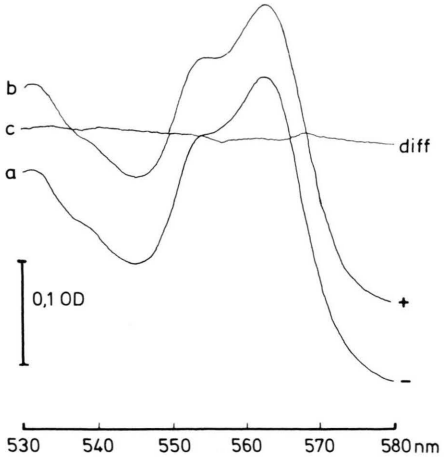

Fig. 6. Effect of stigmatellin on the spectrum of reduced cytochrome *bc1*-complex from beef heart. The complex (4 μM in cytochrome *c1*) was reduced by excess dithionite and its spectrum was measured as described under Methods, before (curve a, −) and after (curve b, +) addition of stigmatellin (80 μM final concentration). Curve c (diff) shows the difference caused by the addition of stigmatellin.

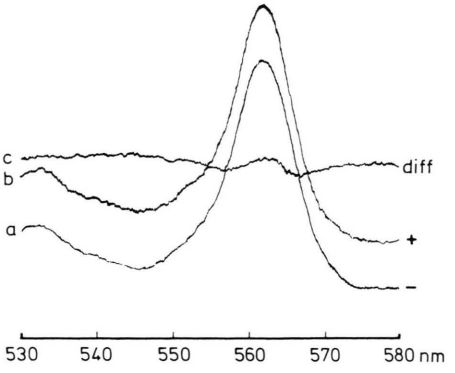

Fig. 8. Effect of stigmatellin on the spectrum of high-potential cytochrome *b* from beef heart. The spectrum in the absence of stigmatellin (curve a, −) shows the difference between spectra recorded at +35 and +80 mV ambient redox potential, the spectrum in presence of stigmatellin (curve b, +) shows the difference between spectra recorded at +50 and +100 mV. The peaks have been normalized to the same height. The effect caused by addition of stigmatellin is shown in curve c (diff).

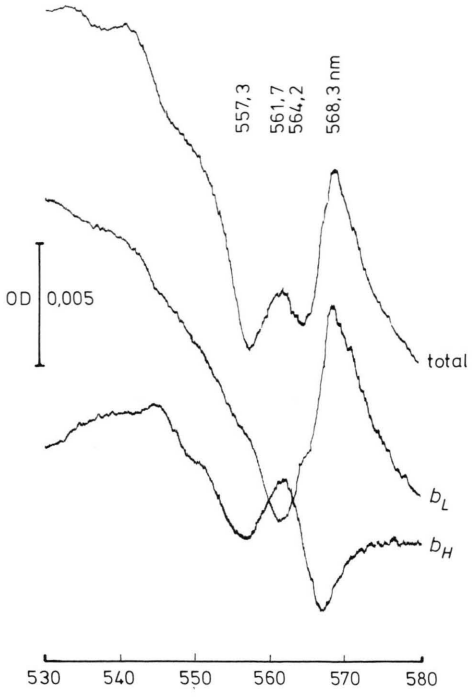

Fig. 9. Effects of stigmatellin on cytochrome *b* at expanded scale. The top spectrum shows the total change, and corresponds trace c of Fig. 6 in expanded form. Characteristic wavelengths of this change are indicated on top. The curve denoted b_H corresponds to the effect of stigmatellin expected for total high-potential cytochrome *b* (the change observed was normalized to half of total cytochrome *b* observable). The curve denoted b_L shows the same for total low-potential cytochrome *b*.

It may be significant that stigmatellin causes a decrease of splitting of the split heme absorptions, which correspond to the high-potential heme in cytochrome *b6*, but to the low-potential heme in cytochrome *b*, and that it causes a narrowing of the rather symmetrical absorptions, corresponding to the respective other two forms. In this context it may also be noteworthy that the split absorptions of the hemes in cytochrome *b* or *b6* correspond to almost the same redox potentials of about −50 mV, while the symmetric absorptions come from either the higher − (+90 mV for cytochrome *b*), or from the lower potential form (−170 mV for cytochrome *b6*, see ref. [16]).

The effects of stigmatellin on the absorptions of the individual hemes in cytochrome *b*/*b6* have been resolved with the assumption that the extinction coefficients are not changed by the inhibitor, what may not be valid. Therefore redox titrations through the whole range of potentials have been performed in presence and absence of the inhibitor. No major change in the relative contributions of the two hemes to total absorption of cytochrome *b*/*b6* has been found. The estimated redox potentials are presented in Table I. It can be seen that all hemes with one exception, including the ones of cytochrome *c1* or *f*, are affected by the inhibitor. All the changes are small compared to the dramatic effect on the Rieske FeS-center [4, 8]. The redox potentials are increased or unchanged by the inhibitor in the mitochondrial complex, but are decreased in the chloroplast complex. Despite the opposite direction, it is again noteworthy that the effect is greater on the high-potential heme of cytochrome *b* and *b6*, which is the heme distal to the quinol-oxidation site.

Our observation that stigmatellin affects all four redox centers of the cytochrome *bc1*/*b6f*-complexes, *i.e.* the FeS-center [4, 8] and all three hemes, can be explained in two ways. Either the four centers are very close to each other, or stigmatellin changes the conformation of the complexes. We favor the second explanation, because of evidences for a change to a tighter conformation of the mitochondrial complex by antimycin [22, 23], which, like stigmatellin [5], binds very strongly and also causes a red-shift of cytochrome *b* [10]. In the case of antimycin this shift is thought to originate from the high-potential heme at the quinone-reduction site [4, 10]. However, our own preliminary results indicate that antimycin, like stigmatellin, affects the absorption and the redox potential of both hemes of cytochrome *b*.

Table I. Effects of stigmatellin on the redox potentials of the hemes in cytochrome *bc1*/*b6f*-complexes. Redox titrations were carried out as described under Methods. Redox potentials were estimated following ref. [14, 15]. In the last column the changes caused by stigmatellin are given.

Component	Redox potential [mV]		
	Control	+ Stigmatellin	Difference
bc1: *c1*	+ 254	+272	+18
b_H	+97	+123	+26
b_L	−32	−32	±0
b6f: *f*	+350	+325	−25
$b6_H$	−45	−70	−25
$b6_L$	−140	−155	−15

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