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1 Individual heme *a* and heme *a*<sub>3</sub> contributions to the Soret  
2 absorption spectrum of the reduced bovine cytochrome *c* oxidase

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24  
25 **CRedit author statement**

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27 analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review &  
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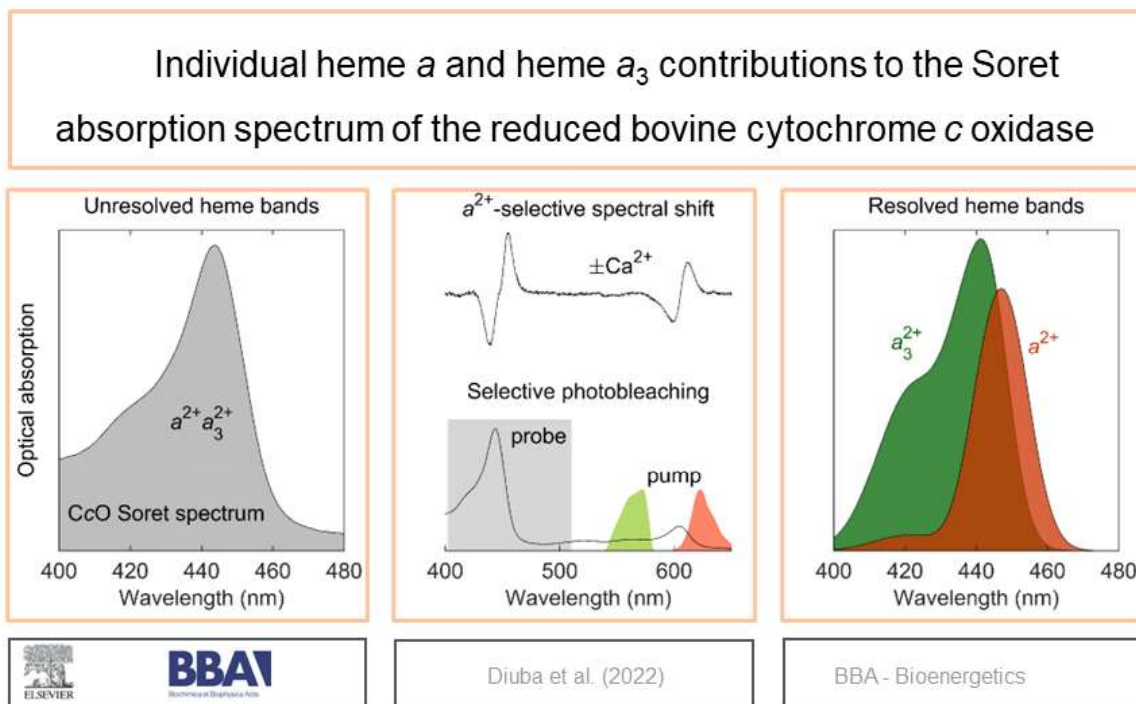
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39 relationships that could have appeared to influence the work reported in this paper.

40

41

**Graphical abstract:**



45           **Abstract**

46           Bovine cytochrome *c* oxidase (CcO) contains two hemes, *a* and *a*<sub>3</sub>, chemically identical  
47 but differing in coordination and spin state. The Soret absorption band of reduced *aa*<sub>3</sub>-type  
48 cytochrome *c* oxidase consists of overlapping bands of the hemes *a*<sup>2+</sup> and *a*<sub>3</sub><sup>2+</sup>. It shows a  
49 peak at ~444 nm and a distinct shoulder at ~425 nm. However, attribution of individual  
50 spectral lineshapes to hemes *a*<sup>2+</sup> and *a*<sub>3</sub><sup>2+</sup> in the Soret is controversial. In the present work,  
51 we characterized spectral contributions of hemes *a*<sup>2+</sup> and *a*<sub>3</sub><sup>2+</sup> using two approaches. First, we  
52 reconstructed bovine CcO heme *a*<sup>2+</sup> spectrum using a selective Ca<sup>2+</sup>-induced spectral shift of  
53 the heme *a*<sup>2+</sup>. Second, we investigated photobleaching of the reduced *Thermus thermophilus*  
54 *ba*<sub>3</sub>- and bovine *aa*<sub>3</sub>-oxidases in the Soret induced by femtosecond laser pulses in the Q-  
55 band. The resolved spectra show splitting of the electronic B<sub>0x</sub> -, B<sub>0y</sub>-transitions of both  
56 reduced hemes. The heme *a*<sup>2+</sup> spectrum is shifted to the red relative to heme *a*<sub>3</sub><sup>2+</sup> spectrum.  
57 The ~425 nm shoulder is mostly attributed to heme *a*<sub>3</sub><sup>2+</sup>.

58  
59           **Keywords:** Cytochrome *c* oxidase, absorption spectra, femtosecond pump-probe  
60 spectroscopy.

61  
62           **Abbreviations:**

63           CcO, cytochrome *c* oxidase

64           FWHH, full width at half-height

65           MCD, magnetic circular dichroism

66           SMR, succinate: menaquinone reductase

67  
68           **Highlights:**

- 69           • Bovine CcO *a*<sup>2+</sup> Soret band is a split electronic 447.5-nm peak with a minimal  
70           short-wavelength satellite
- 71           • *a*<sup>2+</sup> α-band is also split
- 72           • *a*<sub>3</sub><sup>2+</sup> Soret band is a split electronic 441-nm peak with a short-wavelength satellite
- 73           • Ratio of integrals under *a*<sup>2+</sup> and *a*<sub>3</sub><sup>2+</sup> Soret bands is approximately 1 to 2

## 75 1. Introduction

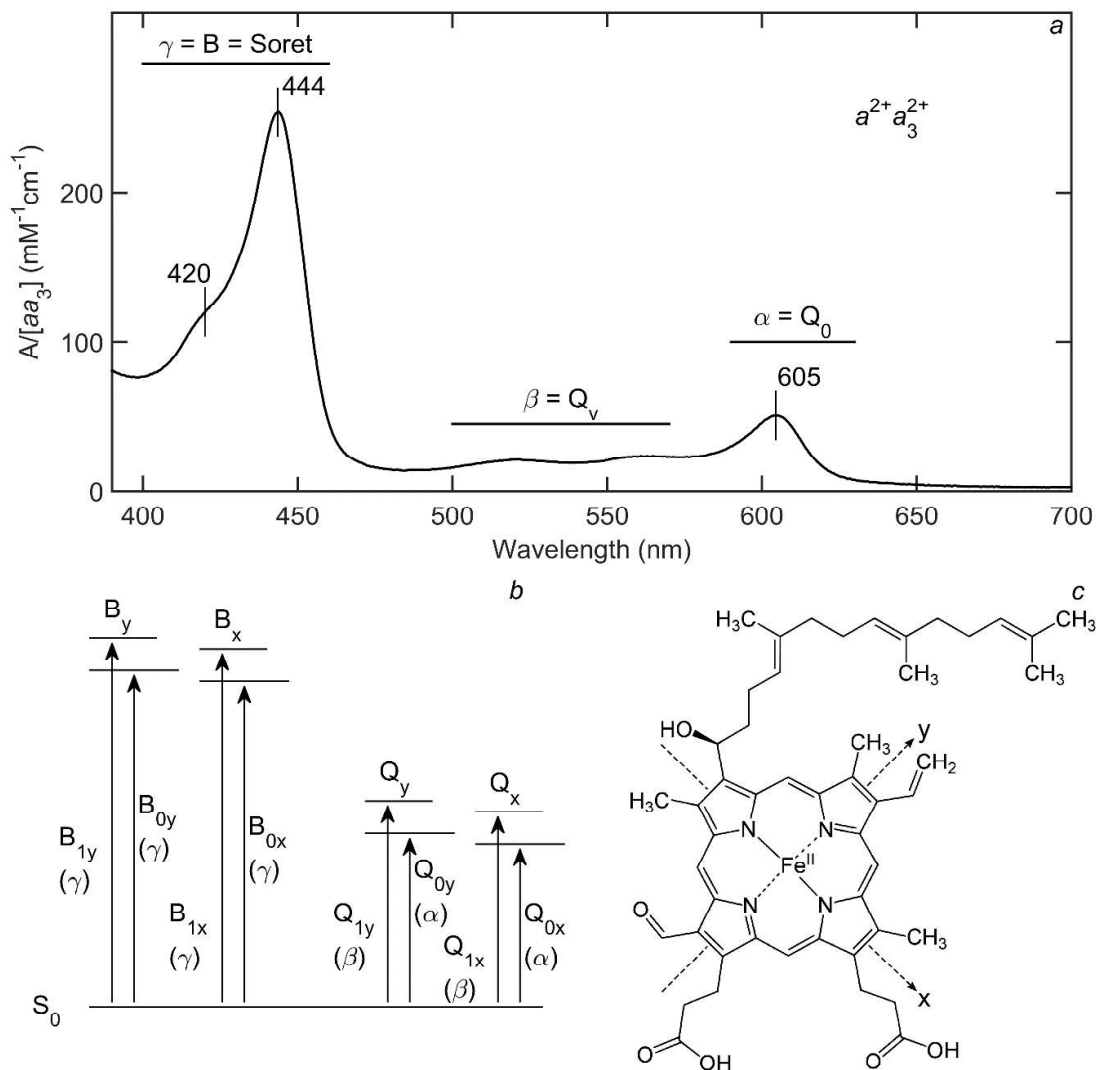
76 In mitochondrial and some bacterial respiratory chains,  $aa_3$ -type cytochrome *c* oxidase  
77 (CcO) catalyzes oxidation of ferrous cytochrome *c* by oxygen, converting redox potential  
78 energy to the transmembrane difference of proton electrochemical potentials,  $\Delta\mu\text{H}^+$ . The  
79 exergonic electron transfer coupled to proton translocation across the membrane [1] via  
80 proton channels [2–6] is mediated by two A-type hemes, low-spin 6-coordinate *a* and high-  
81 spin 5-coordinate  $a_3$ , and two copper centers: binuclear  $\text{Cu}_A$  and mononuclear  $\text{Cu}_B$  [5,7–9].

82 Absorption spectra of hemes *a* and  $a_3$ , sensitive to their redox and spin state, are  
83 routinely used to monitor the reaction. **Fig. 1** shows the absorption spectrum of dithionite-  
84 reduced bovine CcO. The heme absorption spectra (350-700 nm, **Fig. 1a**) display empirically  
85 defined  $\alpha$ -,  $\beta$ - and  $\gamma$ - (Soret) bands corresponding to the electronic and vibronic transitions  
86 from the same ground state to increasingly higher-energy excited states (**Fig. 1b**). Platt's  
87 convention [10] denotes the Soret band as B-band and groups the  $\alpha$ - and  $\beta$ -bands into the Q-  
88 ("visible") band. The  $\alpha$ - and  $\gamma$ -bands comprise two perpendicular electronic transitions each  
89 ( $Q_{0x}$ ,  $Q_{0y}$  and  $B_{0x}$ ,  $B_{0y}$ , respectively) oriented in the porphyrin plane (**Fig. 1c**). Each of the four  
90 purely electronic transitions may have a vibronic satellite due to transition to a non-zero  
91 vibrational state (for example,  $B_{1y}$ , etc). The  $\beta$ -band comprises vibronic satellites of the  $\alpha$ -  
92 band.

93 While heme *a* absorption dominates the Q-band, overlapping heme *a* and  $a_3$  bands with  
94 high and similar intensities challenge spectral interpretation in the Soret. The Soret band of  
95 reduced bovine CcO shows a main peak at 443-444 nm accompanied by a shoulder at ~425  
96 nm (**Fig. 1a**). Our current knowledge of individual absolute spectra of hemes  $a^{2+}$  and  $a_3^{2+}$   
97 relies on Vanneste's indirect derivation [11] based on variable-valence vs unliganded state  
98 spectral comparison referenced to the action spectrum of CO photodissociation from yeast  
99 CcO as the absolute spectrum of CO-bound heme  $a_3^{2+}$ . A 444-nm peak/~428-nm shoulder  
100 pair and a symmetric 442.5-nm peak (with a weak shoulder at ~420 nm) are therein  
101 respectively attributed to heme  $a^{2+}$  and heme  $a_3^{2+}$  (Fig. 5 in ref. [11] and **Fig. A.1**); these  
102 spectra are included in the textbooks and monographs (e.g. [12]). However, bovine CcO  
103 reduction kinetics analysis led Orii [13] to essentially opposite assignment of a featureless  
104 ~445-nm peak and the asymmetric band with a short-wavelength shoulder at ~428 nm to  
105 hemes  $a^{2+}$  and  $a_3^{2+}$ , respectively (see **Fig. A.1**).

106 In this work, we resolve individual absolute absorption spectra of the reduced bovine  
107 CcO hemes *a* and  $a_3$  using two yet different approaches. First, we reconstructed the heme  $a^{2+}$

108 spectrum from  $\text{Ca}^{2+}$ -induced spectral shift. Second, we selectively excited hemes  $a^{2+}$  and  $a_3^{2+}$   
 109 with femtosecond laser pulses and measured photobleaching spectra of the two hemes. Both  
 110 approaches showed  $B_{0x}/B_{0y}$ -splitting in both hemes, heme  $a^{2+}$  transitions red-shifted relative  
 111 to heme  $a_3^{2+}$  transitions, and the  $\sim 425$  nm shoulder attributed mostly to heme  $a_3^{2+}$  spectrum.  
 112



113 **Fig. 1. Absorption bands of reduced cytochrome c oxidase.** (a) experimental  
 114 absorption spectrum of “activated” (see 2.1) CcO (1.7  $\mu\text{M}$ ) bovine CcO reduced by dithionite.  
 115 (b) electronic ( $Q_{0x}$ ,  $Q_{0y}$  and  $B_{0x}$ ,  $B_{0y}$ ) and first vibronic ( $Q_{1x}$ ,  $Q_{1y}$  and  $B_{1x}$ ,  $B_{1y}$ ) transitions from  
 116 the ground state  $S_0$  corresponding to the  $\alpha$ -,  $\beta$ - and  $\gamma$ -absorption bands for single hemes. (c)  
 117 orientation of the x,y-transitions in the plane of porphyrin ring of heme A.  
 118

119 **2. Materials and methods**

120 *2.1. Reagents and preparations.* *aa*<sub>3</sub>-type CcO was isolated from bovine heart  
121 mitochondria according to [14] modified as described in [15] and dissolved in 25-50 mM  
122 HEPES containing 0.05% DM. The concentration of the enzyme was determined from  
123 “dithionite-reduced *minus* oxidized” difference absorption spectra using an extinction  
124 coefficient  $\Delta\epsilon_{605-630}$  of 27 mM<sup>-1</sup>cm<sup>-1</sup>. Before KCN binding, CcO was “activated” as follows.  
125 Argon-deoxygenized ~30  $\mu$ M CcO in 100mM HEPES/tris pH 8.0 was reduced by a small  
126 amount of dithionite in the presence of 20  $\mu$ M of hexaammineruthenium (Ru(NH<sub>3</sub>)<sub>6</sub><sup>3+</sup>; RuAm)  
127 for 30 minutes until full reduction. Dithionite and RuAm were subsequently removed using  
128 centrifugal filter devices (Amicon Ultra-4, Millipore). *ba*<sub>3</sub>-type CcO purified from *Thermus*  
129 *thermophilus* was prepared as described [16]. The concentration of the *ba*<sub>3</sub> CcO was  
130 determined using extinction coefficient  $\Delta\epsilon_{560-590} = 26$  mM<sup>-1</sup>cm<sup>-1</sup> for the absolute spectrum of  
131 the fully reduced oxidase [17]. Succinate-menaquinone reductase preparation was a  
132 generous gift from Prof. Lars Hederstedt (Lund University, Sweden). The concentration of the  
133 enzyme was determined from “dithionite-reduced *minus* oxidized” difference absorption  
134 spectra using an extinction coefficient  $\Delta\epsilon_{558-575}$  of 45 mM<sup>-1</sup>cm<sup>-1</sup> [18].

135 *2.2. Static spectroscopy.* Absorption spectra were recorded in a Cary-300 Bio  
136 spectrophotometer in 1 ml quartz semi-micro cuvettes with a 1 cm optical path. A magnetic  
137 circular dichroism spectrum was recorded at room temperature in a quartz cuvette with a  
138 custom-built dichrograph (effective magnetic field ~ 0.7T for a 1 cm cell). The difference  
139 between direct and reverse magnetic field spectra was calculated to eliminate the contribution  
140 of the natural circular dichroism.

141 *2.3. Femtosecond selective photobleaching of the hemes.* Multicolor pump-probe  
142 spectroscopy was performed with a setup based on a Quantronix Integra-C Ti-Saph oscillator/  
143 amplifier system, delivering ~100 fs, 1.0 mJ pulses centered at 780 nm and operated at a  
144 repetition rate of 500 Hz. Pump pulses with different spectral characteristics (maxima at 623,  
145 608, and 570 nm for the experiments with *aa*<sub>3</sub>; 623 and 570 nm for the experiments with *ba*<sub>3</sub>)  
146 with an energy of ~1  $\mu$ J were generated using a home-built, BBO-based (1 mm thick,  $\theta=29.2^\circ$ )  
147 type I non-collinear optical amplifier (NOPA [19]). The NOPA was pumped with pulses  
148 centered at 390 nm, generated by second harmonic generation of part of the 780-nm  
149 fundamental beam in a 1-mm  $\theta=32^\circ$  BBO crystal, and seeded with white light continuum  
150 generated by focusing part of the 780-nm beam on a 1 mm sapphire plate; the continuum was  
151 subsequently filtered appropriately using selected sharp edge interference filters (Semrock).

152 White light probe pulses were generated by focusing part of the fundamental on a 1 mm-thick  
153 CaF<sub>2</sub> window that was continuously translated perpendicular to the beam. The probe beam  
154 was dispersion-minimized around 435 nm by passing through a prism compressor, and then  
155 separated into the test and reference beams. After passing through the sample, the beams  
156 were dispersed and detected, shot-by-shot, on a CCD camera (Roper Scientific, SPEC-10)  
157 configured as a dual-array detector.

158 To mimic isotropic conditions, the polarization of the pump beam was set at 54.7°  
159 («magic angle») relative to polarization of the probe beam using a polarizer and a half-wave  
160 plate. To vary delay between the pump and probe pulses, the pump beam was passed  
161 through a delay line comprised of a retroreflector mounted on a step-motor driven translation  
162 stage. The 1-mm optical pathlength sample cell was rastered in a Lissajous scanner to  
163 ensure optimal exchange of the sample in the interaction volume. For every type of pump  
164 pulse, 10 spectrokinetics sets were recorded in the range 400-500 nm (spectral resolution ~1  
165 nm) and with temporal steps of 83 fs from -1 to 2 ps and of 2 ps from 2 to 50 ps. Due to  
166 dispersion the effective zero time depended on the wavelength, varying the onset of the  
167 absorption changes for up to 700 fs. We accounted for this dispersion during data processing.

168 *2.4. Preparation of the samples for the femtosecond spectroscopy.* Enzymes were  
169 diluted in 200 mM HEPES/Tris buffer, pH ~ 7, with 0.05% DM dodecylmaltoside and 0.5 mM  
170 Na-EDTA. Quartz cuvettes with a 1 mm optical pathway (“Hellma-Analytics”), type 110-QS  
171 were filled with the sample and hermetically closed with plug-type rubber sleeve stoppers  
172 (“Kimble”, USA). CcO and succinate: menaquinone reductase (SMR) were reduced with a  
173 fresh 0.2M sodium dithionite solution (final concentration ca. 1-5 mM dithionite). To prepare  
174 the dithionite solution, degassed MilliQ was added to degassed dithionite powder into an  
175 argon-filled vial. The sample in the cuvette was degassed with a vacuum pump and the  
176 volume above the sample was saturated with argon. Dithionite was added through the  
177 stopper using a Hamilton syringe, and the volume above the sample was again saturated with  
178 argon. Reduction was considered complete if the absorption spectrum corresponded to the  
179 reduced form (in the case of CcO showing maxima at 444 nm and 605 nm) and stayed  
180 unchanged for at least 10 minutes. The absorption in the Soret band of the reduced form was  
181 0.8-1.0 OD.

182 *2.5. Data analysis.* Global analysis of the pump-induced absorption changes was  
183 performed using Glotaran software [20] to extract the fastest evolution-associated spectra  
184 (EASs) with correction for the instrumental response function, zero-time dispersion and



185 coherent artifacts. Prior to global analysis, noise in the data was reduced using singular value  
186 decomposition. Relaxation included typically three phases with characteristic times of ca. 0.2,  
187 1 and 5 ps, the fastest component representing photobleaching. Additional components did  
188 not improve the fit. Decompositions into gaussians were performed in MATLAB using the  
189 'Interior-point' algorithm [21–23] to minimize the mean square difference between the  
190 experimental and simulated curves.

191

192

### 193 3. Results

194 3.1. *Reconstruction of heme  $a^{2+}$  absorption bands using  $Ca^{2+}$ -induced spectral shift.* In  
195 CcO from vertebrate mitochondria, a specific cation-binding site in subunit I near heme *a* and  
196  $Cu_A$  reversibly binds  $Ca^{2+}$  and  $Na^+$  ions [24–26].  $Ca^{2+}$  red-shifts the entire heme *a* absorption  
197 spectrum by  $\sim 1$  nm [24,27]. The shift is small compared to the linewidths of the heme  $a^{2+}$   
198 absorption bands, so the difference spectrum of the shift represents the differential of the  
199 absolute  $a_3$ -free heme  $a^{2+}$  spectrum, and can be used to derive the heme  $a^{2+}$  absolute  
200 spectrum lineshape [28].

201 We first compared the  $Ca^{2+}$ -induced spectral shift in the fully reduced unliganded  
202 complex  $a^{2+}a_3^{2+}$  and the mixed-valence cyanide complex  $a^{2+}a_3^{3+}$ -CN (**Fig. 2a,b**). In contrast to  
203 absolute spectra (**Fig. 2a**), the  $Ca^{2+}$ -induced difference spectra (**Fig. 2b**) for these two states  
204 are virtually identical both in the Soret and the visible. This finding confirms that heme  $a^{2+}$  is a  
205 unique contributor to the  $Ca^{2+}$ -induced spectral changes. Thus, the non-zero  $a_3^{2+}$  contribution  
206 put forward earlier [24] is not supported by our data obtained using a more stable reduction  
207 system. It also shows that the heme *a* spectrum is not affected by the redox and spin state of  
208 heme  $a_3$ .

209 We chose the  $a^{2+}a_3^{3+}$ -CN complex as the starting point to better separate the  
210 overlapping  $\gamma$ -peaks and minimize any possible interference from heme  $a_3$ . The absolute  
211  $a^{2+}a_3^{3+}$ -CN spectrum (**Fig. 2a**, black) showed a split Soret band with maxima at 442 nm and  
212 430 nm (mainly heme  $a^{2+}$  and heme  $a_3^{3+}$ -CN, respectively) and rather symmetric  $\alpha$ -band at  
213 605 nm.

214 The corresponding difference spectrum (**Fig. 2b**, black) is dominated by narrow 1<sup>st</sup> –  
215 derivative-like features in the Soret and in the visible. The Soret band signal shows  $A_{\min}$  at  
216 438 nm,  $A_{\max}$  at 460 nm and a zero-crossing point at 449 nm, that coincides with neither of  
217 the two maxima in the parent absolute spectrum. An inflection around the zero-crossing point

218 suggests juxtaposition of two overlapping 1<sup>st</sup> derivative-shaped components [24,28]. In the  
219 visible, the difference spectrum shows the zero-crossing point at 605 nm, close to the  
220 maximum of heme  $a^{2+}$ . A small derivative-shaped feature centered around 520 nm may  
221 correspond to a red shift of the  $\beta$ -band of heme  $a^{2+}$ .

222 The integral of this differential spectrum in **Fig. 2b** gives the reconstructed lineshape of  
223 the heme  $a^{2+}$  absolute spectrum (**Fig. 2c**, black). A weak baseline (**Fig. 2b**, green dotted line)  
224 was subtracted from the difference spectrum before integration to avoid negative values in the  
225 reconstructed spectrum. We decomposed the spectral lineshape into a sum of gaussians with  
226 the following assumptions:

227 (1) the Soret and  $\alpha$ -band are comprised of two individual electronic transitions (cf. **Fig.**  
228 **1b,c**);

229 (2) the x and y transitions can be degenerate or non-degenerate in energy;

230 (3) The x and y transitions of the same band must have equal linewidths, but their  
231 intensity ratio may vary;

232 (4) Bands in 500-580 nm region are approximated by two gaussians of variable  
233 linewidths.

234 The reconstructed heme  $a^{2+}$  Soret band with a maximum at 447.5 nm is symmetric and  
235 much narrower than reported by Vanneste (full width at half-height (FWHH) 18 nm vs 24 nm,  
236 correspondingly [11]). It is described by the sum (**Fig. 2c**, red) of two gaussians of equal  
237 FWHH of 15 nm at 442 nm and 450 nm, and an additional minor component at 420 nm with a  
238 FWHH of 18 nm (red dotted). We assign the gaussians at 442 and 450 nm to two non-  
239 degenerate  $B_y$  and  $B_x$  electronic transitions in heme  $a^{2+}$ , split by 8 nm, respectively.

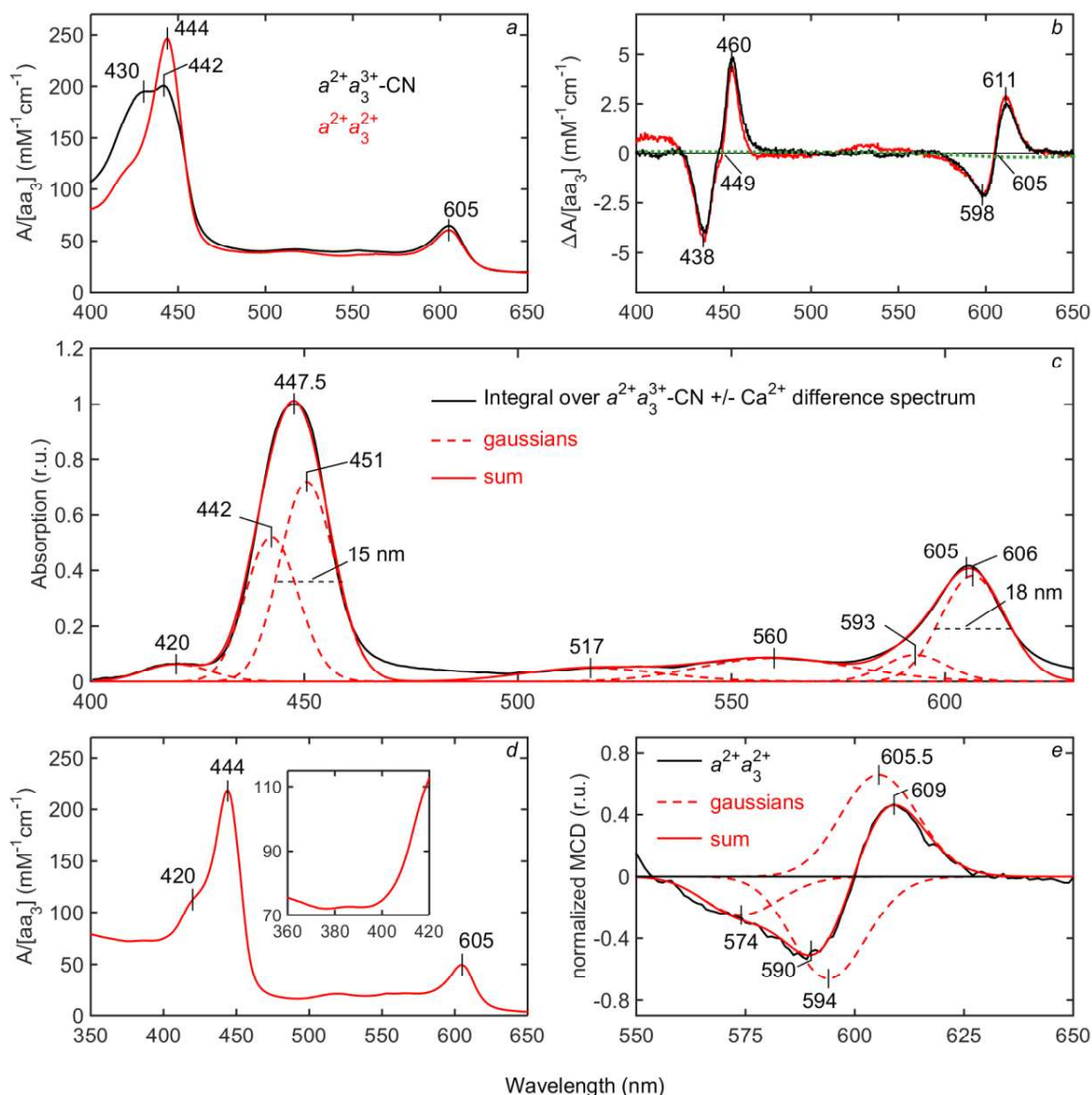
240 Short-wavelength components of the heme  $a^{2+}$  Soret band could reside, in principle,  
241 below 420 nm. However, most conventional reductants such as dithionite or ascorbate  
242 strongly absorb in the near-UV possibly masking these spectral components. We therefore  
243 attempted to obtain a “clean” spectrum of CcO in the Soret band by bubbling gaseous  
244 hydrogen *in statu nascendi* released by sodium borohydride through the anaerobic solution of  
245 the oxidized enzyme. Hydrogen bubbling resulted in partial (~85%) CcO reduction. The H<sub>2</sub>-  
246 reduced CcO spectrum corrected for the fraction of the oxidized form (**Fig. 2d**) reveals no  
247 additional spectral components below 420 nm.

248 The splitting of the Soret band of heme  $a^{2+}$  implies that the  $\alpha$ -band of the heme should  
249 similarly contain split x- and y- components [29]. The  $\alpha$ -band differential Ca<sup>2+</sup>-induced  
250 spectrum shows certain asymmetry (**Fig. 2b**), and, consistently, the reconstructed  $\alpha$ -band

251 (maximum at 605 nm, FWHH 21 nm) has somewhat extended short wavelength side. The  $\alpha$ -  
252 band fitting requires two gaussians of 17-18 nm FWHH separated by 13 nm (at 593 nm and  
253 606 nm). However, in variance with the Soret band, the x and y components of the  $Q_0$ -band  
254 differ much in intensity, so that the overall maximum at 605 nm almost coincides with the  
255 maximum of the dominating  $Q_{0x}$ -transition at 606 nm.

256 To further explore the proposed  $Q_0$ -band splitting, we recorded room temperature  
257 magnetic circular dichroism (MCD) spectrum of our CcO preparation in the visible range (**Fig.**  
258 **2e**, black). The spectrum with positive and the negative lobes at 609 nm and 590 nm and  
259 zero-crossing at 600 nm is very close to that in [29], except for a slight shift of our spectrum to  
260 the red. The MCD spectrum decomposes into a sum of two gaussians of equal magnitude  
261 and width but of opposite sign corresponding to the individual heme  $a^{2+}$   $Q_{0x}$  and  $Q_{0y}$  terms,  
262 and a third negative gaussian at 574 nm (**Fig. 2e**, red dashed: gaussians, red solid: their  
263 sum). The extrema of the two components are observed at 594 (min) and 605.5 nm (max),  
264 *i.e.*, very close to the maxima of the two components fitted to the heme  $a^{2+}$   $\alpha$ -band (**Fig. 2c**),  
265 supporting their attribution to the  $Q_{0x}$ - and  $Q_{0y}$ - electronic transitions.

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**Fig. 2. Reconstruction of the heme  $a^{2+}$  absorption spectrum using selective  $\text{Ca}^{2+}$ -induced spectral shift.** (a) Absorption spectra of the bovine CcO: black, mixed-valence CN-complex  $a^{2+}a_3^{3+}$ -CN; red, fully reduced unliganded enzyme  $a^{2+}a_3^{2+}$ . To obtain the mixed-valence CN-complex, “activated” (see 2.1) CcO (1.7  $\mu\text{M}$ ) in the basic medium was incubated for 10 minutes with 4 mM KCN and then reduced by 5 mM Tris-ascorbate in the presence of 100  $\mu\text{M}$  TMPD. To produce fully reduced form, CcO was incubated with 5 mM ascorbate and 50  $\mu\text{M}$  RuAm, in a cell covered by a gastight cap. Anaerobic condition followed by full enzyme reduction were achieved after 5-min incubation. (b) Difference spectra of the  $\text{Ca}^{2+}$ -spectral shift of heme  $a$ . The curves show the difference between the absolute spectra of the CcO mixed-valence CN-complex (black) or fully reduced form (red) after the addition of 0.1 mM

278 CaCl<sub>2</sub> and after the subsequent addition of 1 mM EGTA. With the adventitious calcium in the  
279 buffer (~ 30 μM), the Ca<sup>2+</sup> concentration after CaCl<sub>2</sub> addition was ~130-fold the K<sub>d</sub> value [26],  
280 i.e., sufficient to saturate the calcium binding site. (c) Reconstructed lineshape of the heme  
281 a<sup>2+</sup> absorption spectrum. Black, integral over the black curve in *b*. Red, its approximation by  
282 the sum of seven gaussians (dashed red). (d) H<sub>2</sub>-reduced bovine CcO absorption spectrum  
283 corrected for the contribution (~15%) of resting oxidized form. The spectrum shows no  
284 additional shoulder below 420 nm. *Insert*: zoom of the 360-420 nm region. (e) Black, MCD  
285 spectrum of reduced bovine CcO. Red, its approximation with 3 gaussian components  
286 (dashed red). To obtain reduced CcO, 50 μM Ru(NH<sub>3</sub>)<sub>6</sub><sup>3+</sup> and 5 mM ascorbate was added to  
287 12 μM CcO in 0.1M HEPES pH 8.1 with 0.1 mM of added Ca<sup>2+</sup>, 5 minutes prior to the  
288 recording of the spectrum.

289

290

291           3.2. *Individual spectra of hemes in the T. thermophilus ba<sub>3</sub> and mitochondrial aa<sub>3</sub> CcO*  
292 *resolved with photoexcitation spectroscopy.* Independently, we employed a selective  
293 photobleaching approach [30–33] by exciting the hemes by a femtosecond light pump pulse  
294 in the Q band to transiently depopulate the electronic ground state and thus to bleach the  
295 corresponding ground state-based absorption. Spectral changes were followed using a white  
296 continuum probe pulse. Since both Q and B bands originate from the same ground state,  
297 excitation in the Q-band bleaches in the Soret as well. Wide separation of the two bands  
298 prevents interference of the relatively narrow pump pulse in the Q-band with the probe pulses  
299 in the Soret.

300           However, the heme  $a^{2+}$  and heme  $a_3^{2+}$  Q-band overlap and finite intrinsic width of the  
301 pump pulses complicates selective excitation of one heme. Therefore, we turned initially to *T.*  
302 *thermophilus ba<sub>3</sub>-type CcO* belonging to the same heme-copper superfamily as the *aa<sub>3</sub>-type*  
303 *oxidases* but with heme *b* instead of heme *a*. In the *ba<sub>3</sub>-type CcO*, the Q-bands of high-spin  
304 heme  $a_3^{2+}$  (~613 nm) and low-spin heme  $b^{2+}$  (559 nm) are separated better than the Q-bands  
305 of bovine CcO hemes *a* and  $a_3$  (**Fig. 3a,b**). The Soret bleaching only occurs if the pump pulse  
306 overlaps with the visible absorption bands. Pumping at 570 nm is expected to excite both  
307 hemes and results in photobleaching spectrum with maximum at 426 nm (heme  $b^{2+}$  band)  
308 with a weak feature at 443 nm (putative heme  $a_3^{2+}$  band) (**Fig. 3c**). In contrast, the pump  
309 pulse at 623 nm (“623exc” condition) (**Fig. 3b**, red) is expected to selectively excite heme  $a_3^{2+}$   
310 since it does not overlap with the heme  $b^{2+}$  narrow  $\alpha$ -band at 559 nm. Compared to the  
311 spectrum of heme  $a^{2+}$  in the bovine CcO (**Fig. 2c**), 623exc photobleaching spectrum (**Fig. 3d**)  
312 is blue-shifted and strongly asymmetric with extended decay on the high-energy side. Besides  
313 two equal-width (12 nm) gaussians at 435 and 444 nm assigned to the purely electronic  $B_{0y}$ -  
314 and  $B_{0x}$ - transitions, the fitting requires a wider band at ~427 nm that may represent the sum  
315 of vibronic components. The absence of 623exc photobleaching of *Bacillus subtilis* reduced  
316 succinate: menaquinone reductase (SMR) containing heme *b* as the only bleaching target  
317 [34] rules out potential heme *b* contribution to this 427-nm band (**Fig. 3d**).

318           Due to spectral overlap, the photobleaching of individual *aa<sub>3</sub> CcO* hemes cannot be  
319 resolved with single excitation. We therefore used three excitation wavelengths to vary the  
320 heme  $a^{2+}$  vs heme  $a_3^{2+}$  excitation degree. **Fig. 4a** shows dithionite-reduced *aa<sub>3</sub>*  
321 photobleaching spectra obtained by excitation with pump pulses centered at 570, 608 and  
322 623 nm (“570exc”, “608exc” and “623exc”). The 608exc spectrum qualitatively resembles the  
323 CcO photobleaching spectrum reported recently for a similar pump-probe protocol with the

324 excitation at 600 nm [35,36]. The “570exc”, “608exc” and “623exc” spectra differ in amplitude  
 325 since the photoexcitation yield depends on the absorption strength in the region of excitation  
 326 and on the pump energy. However, their lineshapes also differ, suggesting varying degree of  
 327  $a^{2+}$  vs.  $a_3^{2+}$  excitation selectivity. The 623exc lineshape is almost symmetrical with a minor  
 328 shoulder below 430 nm. The shoulder contributes more to 608exc and especially 570exc  
 329 spectra. The maximum of the 570exc spectrum is slightly blue-shifted (see **Fig. 4a**, color  
 330 traits). As the heme  $a_3^{2+}$  ground state absorption spectrum is blue-shifted relative to heme  $a^{2+}$   
 331 spectrum (cf. **Fig. 2c** and **Fig. 3d**), the blue shift of the maximum on the 570exc spectrum  
 332 provides a first indication that heme  $a_3^{2+}$  contributes more to the 570exc spectrum than to the  
 333 608exc and 623exc spectra.

334 To estimate the excitation selectivity, we convoluted the pump pulse positions with the  
 335 Q-bands of the hemes  $a^{2+}$  and  $a_3^{2+}$  (**Fig. 4b**). The spectrum reconstructed from the  $Ca^{2+}$ -  
 336 induced shift assay was used to model the heme  $a^{2+}$  Q-band, assuming that heme  $a^{2+}$   
 337 contributes about 80% of absorption in the  $\alpha$ -band maximum. The heme  $a_3^{2+}$  Q-band was  
 338 modeled by subtraction of the heme  $a^{2+}$  spectrum from the smoothed  $aa_3$  Q-band. The  
 339 resulting  $a_3^{2+}$  spectrum has a maximum at ~600 nm and a shoulder below 580 nm, consistent  
 340 with the reduced minus oxidized heme  $a_3$  spectrum [37] (see also **Fig. A.5**). We then  
 341 estimated the heme  $a^{2+}$  vs. heme  $a_3^{2+}$  selectivity as the convolution of the pulse profile with  
 342 the heme  $a^{2+}$  absorption band divided by a convolution of the same pulse profile with the  
 343 heme  $a_3^{2+}$  absorption band:

$$344 \quad a : a_3 \text{ selectivity} = \frac{\int I_a(\omega) I_{pulse}(\omega)}{\int I_{a_3}(\omega) I_{pulse}(\omega)} \quad (1)$$

345 where  $I_a$  and  $I_{a_3}$  are heme  $a^{2+}$  and heme  $a_3^{2+}$  Q-bands, respectively,  $I_{pulse}$  is the  
 346 corresponding pump pulse profile (570exc, 608exc or 623exc), and the integrals are taken  
 347 over the 15391-19992  $cm^{-1}$  spectral range (500-650 nm).

348 The estimated  $a^{2+}$  vs.  $a_3^{2+}$  excitation yield ratios (**Fig. 4b**) suggest a higher contribution  
 349 of heme  $a^{2+}$  to the photobleaching spectra at 623exc and 608exc and approximately equal  
 350  $a_3^{2+}$  and  $a^{2+}$  contributions at 570exc. However, under any excitation conditions, it was not  
 351 possible to assign the photobleaching spectra entirely to either heme  $a^{2+}$  or  $a_3^{2+}$ . To extract  
 352 “pure” hemes  $a^{2+}$  and  $a_3^{2+}$  spectral lineshapes we performed simultaneous decomposition of  
 353 the photobleaching spectra obtained at all three excitations (**Fig. 4c-e**) with the following  
 354 constraints:

355

356 .(1) The Soret ( $B$ -) band of each heme is modeled by 3 gaussian components: two electronic  
357 transitions ( $B_{0x}$ - and  $B_{0y}$ -) with equal widths plus a putative vibronic satellite of variable width.  
358 The three gaussians describing heme  $a^{2+}$  and heme  $a_3^{2+}$  contributions are shown in **Fig. 4c-e**  
359 by orange and green, respectively.

360 (2) Position and bandwidth in  $\text{cm}^{-1}$  (but not amplitude) of a specific transition within a  
361 heme is identical for all three photobleaching spectra. For example, the heme  $a^{2+}$   $B_{0x}$ -  
362 associated gaussians are centered at the same wavelength and have the same width in each  
363 of the three sets of gaussians but may differ in amplitude.

364 (3) The resulting shape of the spectrum of an individual heme ( $a^{2+}$  or  $a_3^{2+}$ ) is preserved  
365 across the excitation conditions. For example, the sums of the three gaussians attributed to  
366 heme  $a^{2+}$  have the same lineshape for each of the three sets of gaussians but may differ in  
367 amplitude.

368 (4) The photoinduced absorption is simulated by two negative gaussians, one at either  
369 side of the spectra, whose positions and bandwidths in  $\text{cm}^{-1}$  were identical across the  
370 excitation conditions.

371 The “pure” normalized heme  $a^{2+}$  photobleaching spectrum (**Fig. 4f**, solid orange) is in  
372 agreement with the spectrum obtained using  $\text{Ca}^{2+}$ -induced shift approach (**Fig. 4f**, gray, same  
373 as **Fig. 2c**, black). It reveals almost symmetrical lineshape with minor shoulder at 421 nm and  
374 contains two individual gaussian components with maxima at 442.5 nm and 450 nm, with a  
375 bandwidth of 14 nm each. In contrast, the “pure” normalized heme  $a_3^{2+}$  photobleaching  
376 spectrum (**Fig. 4g**) has a pronounced shoulder described by 423 nm band with a 25 nm  
377 width. In both hemes  $a$  and  $a_3$ , the  $B_x$ - and  $B_y$ - electronic transitions are separated by  $\sim 8$  nm.

378 As a consistency check, we attempted to reconstruct the overall  $a^{2+}a_3^{2+}$  Soret band as a  
379 weighted sum of the individual  $a^{2+}$  and  $a_3^{2+}$  contributions. First, we estimated the proportion of  
380 the heme  $a^{2+}$  vs. heme  $a_3^{2+}$  contribution by assuming that the  $a:a_3$  excitation selectivity is  
381 most reliably estimated for 608exc condition, where the pumping is close to the heme  $a^{2+}$   
382 band maximum (**Fig. 4b**). Selectivity modeling suggests that pumping at 608 nm excites  
383 heme  $a^{2+}$  3.15 times stronger than heme  $a_3^{2+}$ . At the same time, the ratio of the areas under  
384 the sum of the gaussians attributed to heme  $a^{2+}$  (orange) to that under the sum of the  
385 gaussians attributed to heme  $a_3^{2+}$  (green) on the 608exc is not 3.15:1 but 1.70:1 (**Fig. 4d**).  
386 This could mean that the contributions of the two hemes to the total extinction are not 1:1 but  
387 rather 0.54:1 (heme  $a^{2+}$ : 35%, heme  $a_3^{2+}$ : 65%). The ratio of the areas under the sums of the  
388 heme  $a^{2+}$  and heme  $a_3^{2+}$ -assigned gaussians for 570exc and 623exc fits (predicted

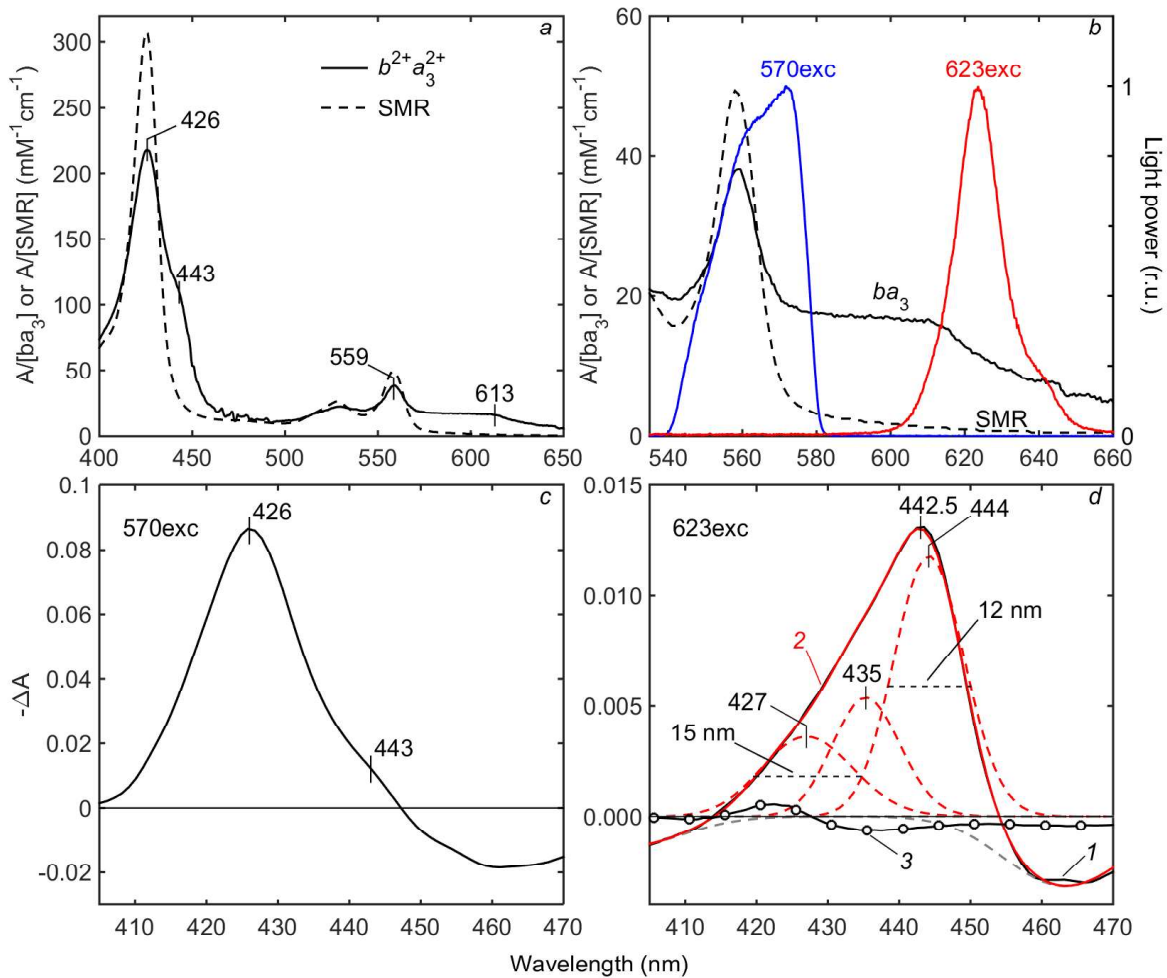


389 selectivities 0.77 and 4.95, respectively) are 0.58:1 and 2.55:1. This gives the following  
390 estimates of the hemes' contributions to the extinction: 43% (heme *a*) and 57% (heme *a*<sub>3</sub>) for  
391 570exc fit; 34% (heme *a*) and 66% (heme *a*<sub>3</sub>) for 623exc fit. The both estimations are fairly  
392 close to that obtained above for the 608exc data which confirms the consistency of our  
393 modeling.

394 Finally, we obtained the reconstructed *aa*<sub>3</sub> Soret lineshape (**Fig. 4h**, red) by adding the  
395 normalized heme *a*<sup>2+</sup> spectrum multiplied by 0.54 to the normalized heme *a*<sub>3</sub><sup>2+</sup> spectrum  
396 multiplied by 1, and normalizing the sum to its maximum. For comparison, the dithionite-  
397 reduced *aa*<sub>3</sub> spectrum is shown, also normalized to its maximum (**Fig. 4h**, gray). The  
398 reconstructed Soret lineshape reproduces the position of the maximum and the shoulder at  
399 the short-wavelength side. We then investigated whether scattering may explain higher  
400 experimental absorption below 430 nm compared to the reconstructed spectrum. For this, we  
401 added  $\sim\lambda^{-4}$  absorption (**Fig. 4h**, dotted dark red curve) to the sum of *a*<sup>2+</sup> and *a*<sub>3</sub><sup>2+</sup> bands. The  
402 maximum-normalized resulting spectrum (**Fig. 4h**, dotted blue curve) is fairly close to the  
403 experimental Soret lineshape. The remaining differences between the reconstructed band  
404 and the experimental *a*<sup>2+</sup>*a*<sub>3</sub><sup>2+</sup> spectrum can be due to imprecise description of the vibronic  
405 satellite(s) by unique gaussian band, unaccounted higher-energy heme transitions, and the  $\beta$ -  
406 band tails. Attempting more precise Soret band reconstruction, as well as determination of  
407 absolute heme absorbance values, is out of the scope of the present work.

408

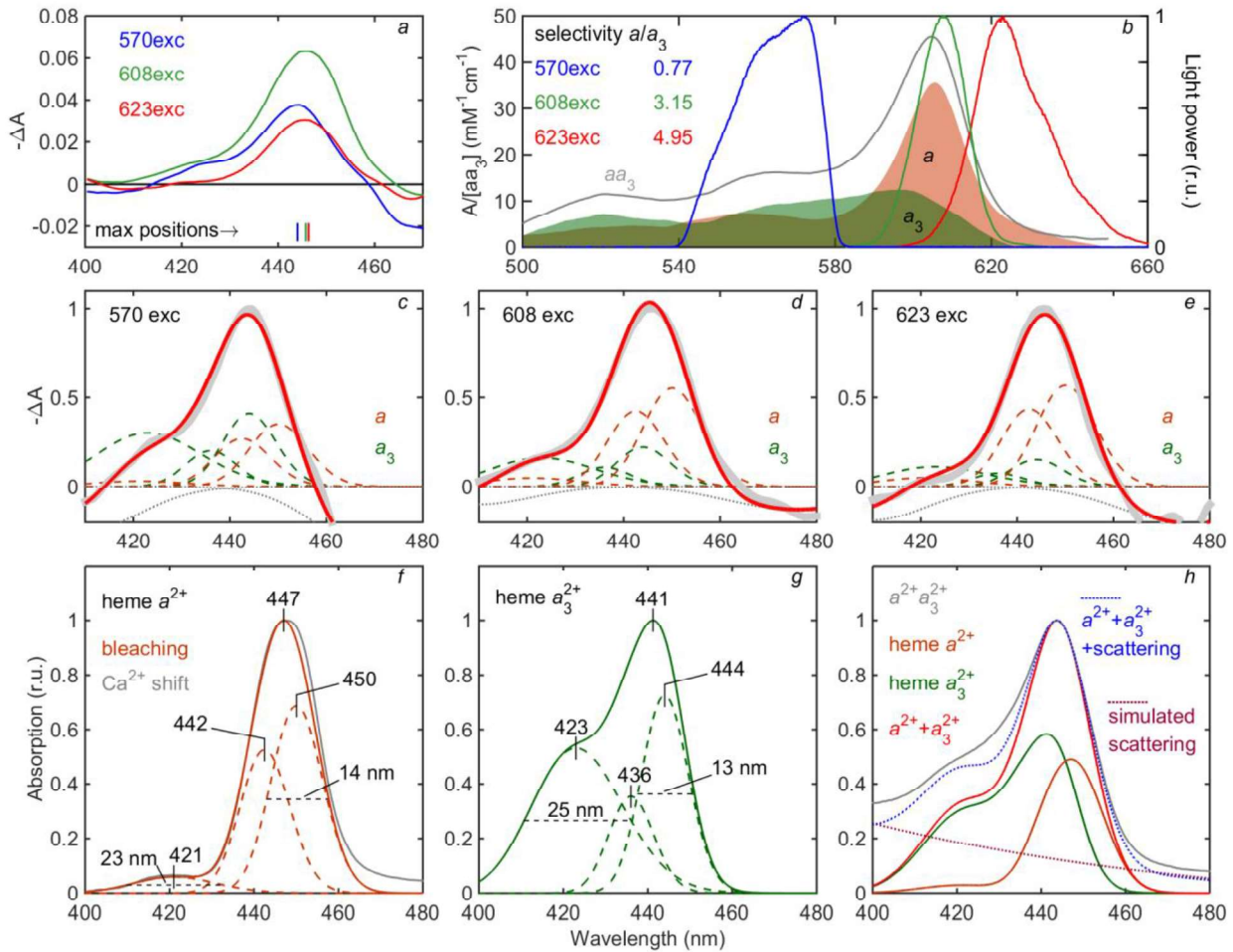
409



410  
 411 **Fig. 3. Photobleaching of reduced  $ba_3$  CcO from *T. thermophilus*.** (a) Dithionite-reduced  
 412 *T. thermophilus*  $ba_3$  (7.9  $\mu\text{M}$ ; solid) and *Bacillus subtilis* succinate: menaquinone reductase  
 413 (SMR, 4.1  $\mu\text{M}$ ; dashed) absorption spectra. (b) Pump laser pulse profiles (right vertical axis)  
 414 centered at 570 nm (blue) and 623 nm (red) relative to the  $\alpha$ -band of the reduced  $ba_3$   
 415 spectrum (black, left vertical axis). The *B. subtilis* SMR spectrum (dashed) does not overlap  
 416 with the 623exc pulse profile suggesting that heme  $b^{2+}$  is not excited by this pulse. (c,d)  
 417 Inverted fastest EASs after pumping at 570exc and 623exc, respectively. In d, Curve 1 (solid  
 418 black), experimental spectrum that has been fitted by 5 gaussians. The 3 positive gaussians  
 419 (dashed red) are assigned to heme  $a_3^{2+}$  photobleaching. Two negative gaussians (dashed  
 420 gray) approximate the increase of absorption following the excitation that likely represent the  
 421 contribution of the electronically excited states (typically weak and diffuse) [38–40]. Curve 2  
 422 (solid red) shows the sum of the 5 gaussians mostly coinciding with the experimental curve 1.  
 423 Curve 3 (black with open circles), control experiment showing that heme  $b^{2+}$  in *B. subtilis*  
 424 SMR is not photobleached by excitation at 623 nm.



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429

**Fig. 4. Photobleaching of reduced bovine CcO.** (a) Inverted fastest EASs after pumping at 570, 608 and 623 nm. The traits at the bottom show corresponding maxima positions. (b) Heme  $a^{2+}$  vs. heme  $a_3^{2+}$  excitation selectivity estimation. Heme  $a^{2+}$  spectrum (red area) reconstructed from the  $Ca^{2+}$ -induced shift (Fig. 2c) was subtracted from the smoothed reduced  $aa_3$  spectrum (gray) to model heme  $a_3^{2+}$  spectrum in the visible range (green area). Heme  $a^{2+}$  was assumed to provide ~80% absorption at the  $\alpha$ -band maximum. The pump pulse profiles for 570exc, 608exc and 623exc are shown in blue, green and red, respectively. Excitation selectivity was estimated using Eqn (1). (c-e) Approximation of the EASs (thick gray lines) by sets of 6 positive (dashed) and 2 negative (dotted) Gaussians. In each fit, three positive Gaussians were attributed to heme  $a_3^{2+}$  (green) and the other three to heme  $a^{2+}$  (orange). The two negative Gaussians describe photoinduced absorption. The sum of the Gaussians is shown by a solid red line. (f,g) Heme  $a^{2+}$  and heme  $a_3^{2+}$  Soret lineshapes (solid lines) determined from the EASs fitting as sums of the three corresponding Gaussians

442 (dashed lines). The lineshapes are normalized to their maxima. In *f*, heme  $a^{2+}$  Soret lineshape  
443 determined with  $\text{Ca}^{2+}$ -induced shift technique (gray) is shown for comparison. (*h*) Bovine  
444  $a^{2+}a_3^{2+}$  Soret lineshape reconstruction. The ratio of the areas under scaled Soret bands of  
445 hemes  $a^{2+}$  and  $a_3^{2+}$  (orange and green) was estimated to be  $a^{2+}:a_3^{2+}=0.54:1$  (see Section  
446 3.2). Red, the sum of the individual  $a^{2+}$  and  $a_3^{2+}$  contributions normalized to its maximum.  
447 Dotted dark red, simulated  $\sim\lambda^{-4}$  scattering. Dotted blue, the sum of reconstructed  $a^{2+}$  and  $a_3^{2+}$   
448 bands and the scattering normalized to its maximum. Gray, Soret band of the dithionite-  
449 reduced bovine CcO normalized to its maximum.

#### 450 4. Discussion

451 **Table 1** resumes heme  $a^{2+}$  and  $a_3^{2+}$  spectral properties reported here and compares  
452 them with previous studies. Only two papers treat explicitly the lineshape of the absolute  
453 spectra of bovine CcO hemes  $a^{2+}$  and  $a_3^{2+}$ : Vanneste's [11] and Orii's [13]. Our spectra  
454 coincide with neither of the two reports but would appear closer to Orii's conclusions.  
455 However, the latter depend critically on the choice of the model parameters and can be  
456 converted into Vanneste's attributions by changing the baseline slope (**Fig. A.2**). It has been  
457 shown by using improved reduction technique that Vanneste's heme  $a_3$  spectra need to be  
458 corrected at least in the  $\alpha$ -region [37]. In the following we will discuss the spectral properties  
459 of the two hemes in detail.

460 *4.1. Soret splitting.* Both hemes  $a^{2+}$  and  $a_3^{2+}$  showed split Soret bands with  $B_{0x}$ - and  $B_{0y}$ -  
461 components separated by 8-9 nm and close electronic transition widths. The heme  $a^{2+}$  Soret  
462 band splitting has been inferred earlier [28,29,41–43], but sometimes misinterpreted as  
463 evidence for two heme  $a$  conformations [42]. The hemes  $a$  and  $a_3$  in CcO have the same  
464 chemical structure - heme A, - but differ in the protein surroundings and in iron coordination.  
465 Therefore, the simplest explanation is that the similar splitting observed for the two hemes is  
466 intrinsic to heme A structure. Indeed, the electronic transition along the pyrrole II  $\rightarrow$  pyrrole IV  
467 axis with the two electron-withdrawing substituents (vinyl and formyl) should differ in energy  
468 from the perpendicular transition along the pyrrole I  $\rightarrow$  pyrrole III axis with electron donating  
469 substituents (methyl, propionate, hydroxyethylfarnesyl group). Conversion of the formyl  
470 substituent to the Schiff-base results in drastic narrowing of the heme A Soret band [44] (see  
471 also **Fig. A.3**) suggesting restoration of degeneracy or at least reduction of splitting. Inherent  
472 heme A splitting is supported by the MCD spectrum of the bis-1-methyl imidazole-6c complex  
473 of heme  $A^{2+}$  showing the same lineshape (except for the position) as the CcO heme  $a^{2+}$  MCD  
474 spectrum [29]. However, it seemingly contradicts the absorption spectra second derivative  
475 analysis (cf. [41] and refs. therein) suggesting that the Soret splitting is specific for heme  $a^{2+}$   
476 in CcO and is not observed in the hexacoordinate (6c-) low-spin (ls-) complexes of isolated  
477 heme  $A^{2+}$  nor in non- $aa_3$ -CcO proteins reconstituted with heme  $a$ .

478 To clarify this issue, we first tested whether deconvolution into gaussians can reveal the  
479 Soret splitting of the isolated heme  $A^{2+}$  spectrum by analyzing the bis-imidazole-heme  $A^{2+}$   
480 spectra reported in [44]. The ls-6c-heme  $A^{2+}$  spectra in solution are broader than in CcO (35  
481 nm in aqueous buffer or 27 nm in  $\text{CH}_2\text{Cl}_2$  vs 18 nm in the CcO protein) and, at this width,  
482 single- and two-gaussian approximations (both including one additional gaussian describing a

483 vibronic shoulder) are equally valid (**Fig. A.3**). Further, simple modeling (**Fig. A.4**) shows that  
484 the ~10-nm splitting of two gaussians with equal heights and widths typical for CcO requires  
485 their FWHH being below ~8 nm to be detected on the second derivative curve. The exact  
486 lineshape of the second derivative depends also on the relative heights of the two gaussians.  
487 Thus, the second-derivative method may resolve the  $B_{0x}$  and  $B_{0y}$  bands of heme *a* in the CO-  
488 complex of reduced CcO, with heme  $a_3$  bands shifted far to the blue (cf. Fig. 1B in [41]) and to  
489 a lesser extent in the fully reduced CcO (Fig. 1A in ref. [41]), but is not expected to resolve the  
490  $B_{0x}$ - and  $B_{0y}$ - bands in the broad spectra of the isolated 6c-heme  $A^{2+}$ .

491 The same applies to the recent second-derivative analysis of the Soret CcO spectra  
492 confirming ferrous heme *a* Soret splitting but arguing against splitting of the ferric hemes *a*  
493 and  $a_3$  Soret bands [45]. On this basis, the authors proposed that the ferric hemes are  
494 symmetric but their reduction changes the protein conformation distorting heme planes and  
495 splitting the  $B_{0x}$ - and  $B_{0y}$ -transitions. However, the Soret bands of the ferric hemes are known  
496 to be broader than those of the reduced hemes and their splitting may be unresolved by the  
497 second derivative assay.

498 We conclude that although not detectable by second-derivative analysis, the inherent  
499 splitting of the heme  $A^{2+}$  Soret band is in the origin of the Soret band splitting of both hemes  
500  $a^{2+}$  and  $a_3^{2+}$ .

501 **4.2. Heme  $a^{2+}$   $\alpha$ -band splitting.** Spectral forms of heme  $a^{2+}$  differing in the  $\alpha$ -peak were  
502 described [46,47] but the possibility of a split  $\alpha$ -band of the heme was not considered in these  
503 studies. Our analysis revealed the presence of a second minor component at 593 nm in  
504 addition to the dominating band at 606 nm (**Fig. 2c**). Their attribution to the split  $Q_{0y}$  and  $Q_{0x}$   
505 bands of heme  $a^{2+}$ , respectively, is supported by MCD spectroscopy that allows for better  
506 resolution of the split bands. The  $\alpha$ -band heme  $a^{2+}$  MCD spectrum can be interpreted as a  
507 sum of two opposite B-terms belonging to the  $Q_{0x}$  and  $Q_{0y}$  transitions in heme  $a^{2+}$  [29]. The  
508 MCD spectrum of our preparation is indeed described as the sum of two identical B-terms of  
509 opposite signs (**Fig. 2e**).

510 The Soret splitting is more extensive than that of the  $\alpha$ -band (381-416 vs 373  $\text{cm}^{-1}$ )  
511 probably due to the larger Soret vs  $\alpha$ -band transition moment implying stronger interaction  
512 with the permanent local electric field [48]. In contrast to the Soret where the  $B_{0x}/B_{0y}$  intensity  
513 ratio is about 1.4 (see **Table 1**), the  $Q_{0y}$  band at 593 nm is much weaker than the  $Q_{0x}$   
514 component at 606 nm (absorption intensity ratio  $Q_{0x}/Q_{0y}$  is ~4). The weaker high-energy ( $y$ )  
515  $Q_0$ -component is typical of metal-porphyrin compounds with split absorption bands [49–53]

516 and agrees with calculations [50,54]. Therefore, the commonly observed  $\alpha$ -peak of heme  $a^{2+}$   
517 at 605 nm largely corresponds to the  $Q_{0x}$  transition of the iron-porphyrin ring of heme  $a^{2+}$ .

518 **4.3. Position of the Soret absorption peaks of the hemes  $a^{2+}$  and  $a_3^{2+}$ .** It is commonly  
519 assumed that the Soret absorption maxima of hemes  $a^{2+}$  and  $a_3^{2+}$  are close to each other  
520 (Vanneste: 444 nm  $a^{2+}$  vs 443 nm  $a_3^{2+}$  [11]; Orii: 445 nm  $a^{2+}$  vs 443 nm  $a_3^{2+}$  [13]). This fact  
521 has been long considered surprising in view of the difference in iron coordination and heme-  
522 protein interaction.

523 Our data, in contrast, suggest that the Soret maxima (447-447.5 nm  $a^{2+}$  vs 441 nm  $a_3^{2+}$ )  
524 and the  $B_{0x}$ -,  $B_{0y}$ -transition energies of hemes  $a^{2+}$  and  $a_3^{2+}$  do actually differ. The heme  $a_3^{2+}$   
525  $B_{0x}$ - and  $B_{0y}$ -transitions are shifted by 6-7 nm to the blue relative to the corresponding  
526 transitions of heme  $a^{2+}$ , in agreement with the blue shift of the reduced minus oxidized  
527 difference spectrum of CcO heme  $a_3$  relative to that of heme  $a$  [37]. In apparent contradiction,  
528 Soret maxima of high spin mono-His-coordinated (5c-hs) cytochromes  $b$  are usually situated  
529 to the red from those of low spin, bis-His-coordinated (6c-ls) cytochromes  $b$  (**Table A.1**).  
530 Congruently, the wide  $\alpha$ -band of 5c-hs 1,2-diMelm-heme  $A^{2+}$  (heme  $a_3^{2+}$  model) locates to the  
531 red of the  $\alpha$ -band of 6c-ls bis-1-Melm-heme  $A^{2+}$  (heme  $a^{2+}$  model) [29,41,44]. The inverted  
532 maxima positions in CcO may be explained by the opposite shifts of the CcO hemes relative  
533 to the corresponding isolated complexes. The spectrum of CcO heme  $a^{2+}$  is red-shifted by 9-  
534 11 nm in the Soret and by 12-16 nm in the  $\alpha$ -band compared to the 6c-ls bis-1-Melm-heme  
535  $A^{2+}$  (**Table 1**). This shift is probably induced by a strong hydrogen bond between the in-plane  
536 oriented heme  $a$  formyl group and the conserved arginine R38<sub>bt</sub> as proposed originally in [55]  
537 and confirmed by mutagenesis of the homologous arginine in bacterial CcOs [56,57]. Indeed,  
538 the replacement of the homologous arginine to methionine causes a 605 nm to 589 nm shift  
539 of the  $\alpha$ -band that would correspond to  $\sim 10$  nm shift in the Soret [56]. On the other hand, the  
540 reduced *minus* oxidized difference spectrum of the CcO heme  $a_3$  appears to be blue-shifted  
541 by about 7 nm compared to that of 5c-hs heme  $A^{2+}$  while preserving the spectral shape ([37];  
542 **Fig. A.5**) suggesting  $\sim 3$ -4 nm blue shift in the Soret.

543 **4.4. B-band short-wavelength shoulder.** Heme  $a_3^{2+}$  shows a pronounced high-energy  
544 shoulder of the Soret peak (**Fig. 4g**), presumably a vibronic satellite of the  $B_0$  transitions.  
545 Isolated 6c-ls heme  $A^{2+}$  also exhibits a shoulder ([44]; **Fig. A.3a,b**). However, no vibronic  
546 satellite can be seen in the spectrum of heme  $a^{2+}$  in CcO, except for a tiny band at 420-421  
547 nm (**Fig. 2c, 4f**). The vibronic  $\beta$ -band shows two peaks at  $\sim 517$  nm and  $\sim 560$  nm. The one at  
548  $\sim 517$  nm [11] or both peaks [58] were attributed to heme  $a^{2+}$  features. Both peaks are red-



549 shifted by  $\text{Ca}^{2+}$  and appear in the integral of the  $\text{Ca}^{2+}$ -induced difference spectrum (**Fig. 2c**).  
550 The first vibrational progression peak, if any, of the  $a^{2+}$  B band would therefore be expected at  
551  $1500\text{ cm}^{-1}$  higher energy than the electronic peak, i.e., at  $\sim 419\text{ nm}$ . Therefore, the small 420-  
552 nm band in the reconstructed heme  $a^{2+}$  spectrum could indeed indicate the presence of a  
553 strongly dampened vibronic mode. The reason of the dampening is not yet clear. However,  
554 the nearly symmetric lineshape of the heme  $a^{2+}$  spectrum is consistent with Soret spectra of  
555 other reduced 6c-Is cytochromes, for example, cytochrome  $c_5$  [59–61], neuroglobin [62–66],  
556 cytochrome  $b_{559}$  [62], superoxide dismutase [62], cytochrome  $b_{560}$  [67], cytoglobin [68], and  
557 SMR (**Fig. 3a**). The absence of the shoulder in the spectrum of the 6c-Is heme integrated into  
558 the protein could be explained by silencing of the corresponding heme plane vibrations by  
559 axial histidines attached to the polypeptide backbone so that out-of-plane movements may be  
560 restricted. Heme  $a_3^{2+}$  and the free bis-imidazole 6c-Is heme  $A^{2+}$  would have this vibrational  
561 mode permitted because the heme is either attached to only one histidine or to axial  
562 imidazoles that are not connected to any larger scaffold.

563 *4.5. Conclusion and outlook.* Upon reinvestigating the decomposition of the reduced  $aa_3$   
564 optical absorption spectrum we propose different contributions of the  $a$  and  $a_3$  hemes that  
565 may resolve long-lasting confusion in the literature. We foresee that this work will be valuable  
566 for (re-evaluation of) spectroscopic studies of the CcO mechanism. Extension of our approach  
567 may come from polarization-sensitive studies in particular on macroscopically ordered  
568 systems and true 2-dimensional pump-probe approaches.

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575 decision to submit the article for publication.

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## 577 **6. Data availability**

578 Numeric data and all scripts used to analyze data and produce figures are downloadable  
579 from Mendeley Data: <https://data.mendeley.com/datasets/dmrkn9pfr/3> [69].

**Table 1.** Spectral characteristics of hemes  $a^{2+}$  and  $a_3^{2+}$  reported in this work and in previous studies

	Soret band						$\alpha$ -band				source
	overall peak	$B_{0y}$	$B_{0x}$	$B_{0y}$ - $B_{0x}$ splitting	$B_{0x}/B_{0y}$ ratio	$B_v$	overall peak	$Q_{0y}$	$Q_{0x}$	$Q_{0y}$ - $Q_{0x}$ splitting	
$a^{2+}$ (bovine)	447.5 (18) 22342 (914)	442 (15) 22609 (741)	451 (15) 22193 (741)	8 416	1.4	420 (18) 23808 (1037)	605 (21) 16517 (567)	593 (17) 16862 (489)	606 (18) 16489 (489)	13 373	This work (Fig.2c,4f) ( $Ca^{2+}$ -shift)
$a^{2+}$ (bovine)	447 (18) 22346 (880)	442.5 (14) 22599 (705)	450 (14) 22218 (705)	7.5 381	1.3	421 (23) 23753 (1295)					This work (Fig.4f) ( $a_{a_3}$ photobleaching)
$a_3^{2+}$ (bovine)	441 (30) 22664 (1608)	436 (13) 22936 (659)	444 (13.0) 22523 (659)	8 413	2.0	423 (25) 23641 (1413)					This work (Fig.4g) ( $a_{a_3}$ photobleaching)
$a_3^{2+}$ ( $ba_3$ )	442.5 (19) 22599 (965)	435 (11) 22972 (589)	444 (12) 22515 (589)	9 457	2.2	427 (15) 23419 (824)					This work (Fig.3d) ( $ba_3$ photobleaching)
$a^{2+}$ (bovine)		442-443	450-451	7-9							Fig.1 in [41]
$A^{2+}$ bis-Im in SDS	438 (35) 22831 (1884) extended on blue						593 (39) 16863 (1104)				Fig.6 in [41]
bis-Im $A^{2+}$ Is aqueous buffer	440 (35) 22726 (1841)	436 (22) <sup>a</sup> 22919 (1177)	446 (23) <sup>a</sup> 22428 (1177)	10 <sup>a</sup> 491	1.2 <sup>a</sup>	416 <sup>a</sup> 24033	597 (57) 16750 (1644)				Fig.3B in [44] and Fig. A.3b of present paper
bis-Im $A^{2+}$ Is $CH_2Cl_2$	438 (27) 22830 (1419)	435 (22) <sup>a</sup> 22989 (1177)	442 (23) <sup>a</sup> 22604 (1177)	7 <sup>a</sup> 385	1.0 <sup>a</sup>	414 <sup>a</sup> 24131	592 (41) 16892 (1182)				Fig.4B in [44] and Fig. A.3d of present paper
Bis- <i>n</i> -butylamine $A^{2+}$ Is Schiff base derivative aqueous buffer	427 (19) 23419 (1023)	425 (16) <sup>a</sup> 23512 (895) 428 (17) <sup>b</sup> 23374 (922)	430 (17) <sup>a</sup> 23256 (895)	5 <sup>a</sup> 256	1.0 <sup>a</sup>	407 <sup>a</sup> 24599 409 <sup>b</sup> 24471	573 (29) 17452 (899)				Fig.3B in [44] and Fig. A.3e,f of present paper
Bis- <i>n</i> -butylamine $A^{2+}$ Is Schiff base derivative $CH_2Cl_2$	428 (18) 23364 (988)	427 (16) <sup>a</sup> 23398 (875) 429 (16) <sup>b</sup> 23326 (870)	430 (16) <sup>a</sup> 23256 (875)	3 <sup>a</sup> 142	1.0 <sup>a</sup>	410 <sup>a</sup> 24399 411 <sup>b</sup> 24331	575 (31) 17391 (958)				Fig.4B in [44] and Fig. A.3g,h of present paper
Bis-1-Melm $A^{2+}$ Is $CH_2Cl_2$ (MCD)		430 <sup>c</sup> 23249	442 <sup>c</sup> 22602	12 647			574 <sup>c</sup> 17435	599 <sup>c</sup> 16706	25 729		Fig. 2 in [29]
Bis-1-Melm $A^{2+}$ Is $CH_2Cl_2$ (absorption)							588 (32) 17001 (935)				Fig. 7 in [29]
1,2-diMelm $A^{2+}$ Is $CH_2Cl_2$ (absorption)							606 (139) 16500 (4493)				Fig. 7 in [29]

581 Format: position (full width at half-height); upper row: nm, lower row:  $cm^{-1}$ ; <sup>a</sup> 3-gaussian fit in Fig. A.4; <sup>b</sup> 2-gaussian fit in Fig. A.4; <sup>c</sup> positions of the two opposite extrema in

582 the MCD spectrum

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