Chromaffin granule membranes contain at least three heme centers: direct evidence from EPR and absorption spectroscopy

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Abstract Low-temperature electron paramagnetic resonance (EPR) spectroscopy, circular dichroism and two-component redox titration have previously provided evidence for two different ascorbate-reducible heme centers in cytochrome b_{561} present in chromaffin granule membranes. These species have now been observed by room and liquid nitrogen temperature absorption spectroscopy. The visualization of these heme centers becomes possible as a consequence of utilizing chromaffin granule membranes prepared by a mild procedure. Additionally, a new redox center, not reducible by ascorbate, was discovered by both EPR and absorption spectroscopy. It constitutes about 15% of the heme absorbance of chromaffin membranes at 561 nm and has EPR characteristics of a well-organized highly axial low-spin heme center (thus making it unlikely that it is a denatured species). This species is either an alternative form of one of the hemes of cytochrome b_{561} that has a very low redox potential or a *b*-type cytochrome distinct from b_{561}.

Key words: Chromaffin granule; Cytochrome b_{561}; Absorption spectroscopy; Electron paramagnetic resonance; Hemoprotein; Ascorbic acid

1. Introduction

Cytochrome b_{561} present in chromaffin granules (CG) of specialized secretory cells in the adrenal medulla is a transmembrane protein playing a central role in supplying electrons for norepinephrine synthesis by shuttling them from cytoplasmic ascorbate to dopamine β-hydroxylase located within the vesicles [1].

Based on an apparent 1:1 heme-to-protein ratio in the purified protein [2–5] and in CG membranes (CGM) [6], cytochrome b_{561} was for a long time considered to be a single-heme-containing protein. In 1989 Esposti et al. [7] proposed the first model of the transmembrane structure of cytochrome b_{561} as a di-heme protein based on sequence analysis and the following data: (i) redox titrations of cytochrome b_{561} are characterized by a non-linear Nernst plot [6,8,9]; (ii) circular dichroism spectra study of CGM [7] revealed an intra-molecule heme–heme interaction. This model was supported by the observation of two different types of electron paramagnetic resonance (EPR) signals in membranous cytochrome b_{561}: a relatively conventional low-spin signal with g_z near 3.1, similar to the EPR signal of cytochrome b_5, and a second signal with $g_z = 3.70$ of the HALS (highly axial low-spin) type, similar to mitochondrial cytochrome $b_L b_H$ [10,11]. Subsequently Okuyama et al. [12] came to a similar model based on sequence comparisons.

In 1997 Tsubaki et al. reported the purification of cytochrome b_{561} containing two hemes per molecule of protein [13]. The EPR spectrum of the isolated protein exhibited two types of signal similar to those observed with the membranous cytochrome [10,11]. In a series of subsequent papers Tsubaki and his colleagues have shown that two heme centers of cytochrome b_{561} have different sensitivity to the denaturing agents [12] and have functionally distinct roles in reacting with ascorbate and the ascorbate radical [14,15].

As the two heme centers in cytochrome b_{561} possess such distinct physico-chemical properties it is reasonable to expect that they should exhibit different optical absorption spectra but thus far there have been no reports in which clearly distinguishable spectral forms could be associated with the high- and low-potential hemes of b_{561}. Apps et al. [6] found that the absorption spectrum of the α-band of ferrocytochrome was clearly asymmetric with a maximum at 561 nm and shoulder at 556 nm but assigned both these spectral features to a single cytochrome species. Only one group, Kamensky et al., reported that while both hemes exhibit identical maxima in their absorption spectra, their lineshapes are distinctly different [10]. Accordingly, they interpreted their data as an indication for the presence of two independent heme centers within a single cytochrome.

We hypothesized that the lack of convincing differences in the individual absorption spectra of two heme centers of membranous cytochrome b_{561} stemmed from the elaborate and time consuming procedures for the preparation of CGM employed for spectroscopic studies by all investigators [6,8,10,16–19]. For this report we utilized CGM prepared according to an alternative procedure that is both mild and rapid [20,21]. We demonstrate that the α-bands of the two cytochrome b_{561} heme centers are different, markedly so when recorded at 77 K.

Additionally and unexpectedly, we discovered a component of cytochrome *b* that has an absorption spectrum and EPR signal distinct from both heme centers of b_{561} described pre-

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Abbreviations: CG, chromaffin granules; CGM, chromaffin granule membranes

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viously. This fraction is responsible for approximately 15% of the optical absorption at 561 nm, and is not reducible by ascorbate, the natural reductant for cytochrome b_{561} [22]; it is presumed to be either a modified form of the $g_z = 3.70$ center having a very low redox potential or a previously unknown *b*-type cytochrome present in the membrane of CG.

2. Materials and methods

CG were isolated from bovine adrenal medulla according to [23]; all solutions were supplemented by 25 mg/l PMSF [4]. The membrane fraction was obtained according to [21] with modifications. Approximately 400–500 mg of frozen CG were homogenized after thawing in 360 ml of lysing media (10 mM HEPES, 0.1 mM EDTA, 3.75% (v/v) glycerol, pH 7.0) and incubated at 4°C with gentle stirring for 45 min. The suspension was spun at $100\,000 \times g$ for 30 min in a Beckman model 35 rotor. The sediment was homogenized in lysing media and loaded onto a Sephadex G-25 column (2×30 cm) that was pre-equilibrated with the same media and allowed to flow by gravity. 7.5 ml of eluate was layered over a 3 ml pad made from lysing media supplemented with 0.9 M sucrose [17], and spun at $200\,000 \times g$ for 1.5 h in a Beckman SW-41 rotor. The membrane fraction was collected from the interface, diluted with 60 ml lysing buffer without sucrose and incubated on ice for 30 min with gentle stirring. The purified membranes were then concentrated by centrifugation at $100\,000 \times g$ for 30 min in the model 35 rotor. The yield was approximately 10–15% of initial CG.

Absorption spectra were collected at 0.04 nm intervals using an IBM 9430 UV-Vis spectrophotometer (2 nm spectra bandwidth) for room temperature experiments and at 0.09 nm intervals using an SLM-Aminco DW-2000 (0.1 nm spectra bandwidth) equipped with a low-temperature accessory for spectroscopic measurements at liquid nitrogen temperature. Cuvettes with 10 mm and 2 mm pathlengths were used for spectra collection at room temperature and 77 K, respectively. The medium for all experiments was composed of HEPES, 50 mM, pH 7.2; EDTA, 0.1 mM and glycerol, 18% (v/v). For room temperature experiments absolute spectra were collected after graded additions of ascorbate with a maximum level of reduction achieved by addition of a few grains of dithionite. The addition of 60 and 100 mM sodium ascorbate was achieved using solid. For experiments at 77 K a stock suspension was prepared in advance in a quantity sufficient for the whole series of spectra. A portion of membrane suspension was transferred to a cuvette and incubated with the appropriate amount of ascorbate for 5 min, then frozen in liquid nitrogen and the absolute spectrum recorded. The next level of reduction was achieved by reducing a fresh portion of the same stock using a larger amount of ascorbate. EPR samples were prepared by plunging the EPR tube into a dry ice–acetone mixture; freezing was completed within approximately 1 s. Difference spectra were calculated from original data using the data analysis and graphing program Igor Pro (Wavemetrics). EPR spectra were collected using a Varian E-6 spectrometer equipped with an Air Products liquid helium thermostat.

3. Results

We collected room temperature absolute absorption spectra of CGM following gradual reduction by ascorbate and subtracted from each of them the spectrum of a sample of oxidized CGM (Fig. 1A). It is apparent that the spectra recorded during the early stages of the titration are more asymmetric than those recorded towards its end; this can only be possible if several species are present, and their absorption spectra are different.

The spectra of the individual components calculated subsequently (Fig. 1B) are divided into a high-potential species (***a***, the difference between spectra ***2*** and ***1*** of Fig. 1A) and a low-potential species (***b***, the difference between spectra ***8*** and ***7*** of Fig. 1A). Both species exhibit an α-band that is clearly split (i.e. consists of two transitions with different intensities leading to a peak and a shoulder) but the positions of the extrema are different (Table 1).

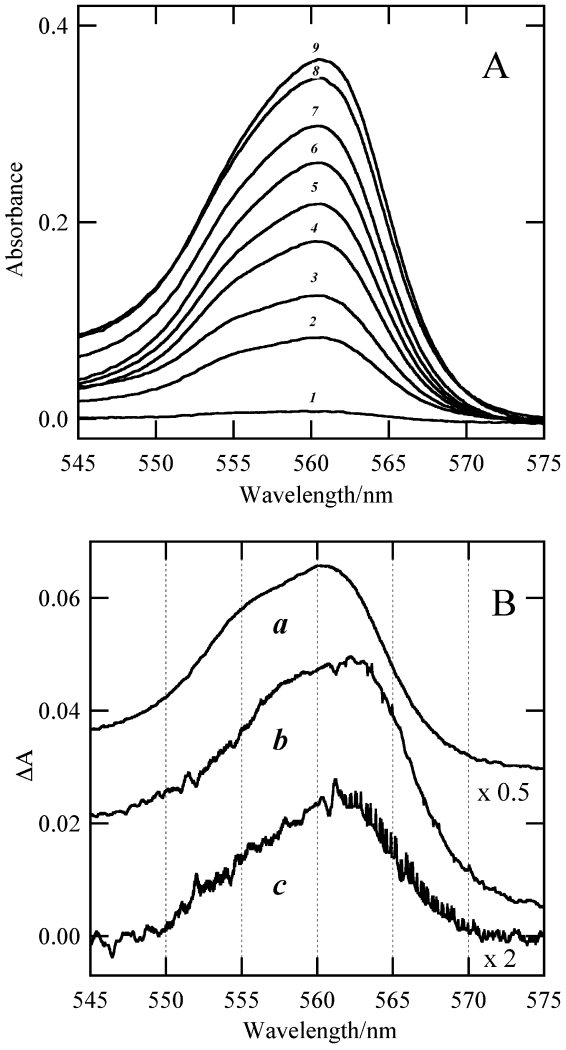

Fig. 1. Reduced minus oxidized difference absorption spectra of CGM collected at room temperature (A) and spectra of individual heme centers deduced from a reductive titration (B). A: CGM were dissolved in the medium containing 50 mM HEPES, 0.1 mM EDTA, 18% glycerol, pH 7.2, placed into a standard cuvette with a 10 mm pathlength and either oxidized by the addition of 8 μM ferricyanide or reduced by the following additions of reductant (all concentrations are final): (***1***) endogenous reductant; (***2***) 25 μM ascorbate; (***3***) 70 μM ascorbate; (***4***) 125 μM ascorbate; (***5***) 250 μM ascorbate; (***6***) 1 mM ascorbate; (***7***) 10 mM ascorbate; (***8***) 100 mM ascorbate; (***9***) dithionite. To correct for a sloping baseline the spectrum of the oxidized sample was subtracted from the spectrum of each reduced sample. B: Absorption spectra of (***a***) high-potential heme center; (***b***) low-potential heme center; and (***c***) ascorbate non-reducible heme center, calculated as the difference between spectra ***2*** and ***1***; ***8*** and ***7***; ***9*** and ***8*** (A), respectively. Note that the amplitude of spectrum (***a***) was scaled down by a factor of 0.5 and that of spectrum (***c***) scaled up by a factor of 2.

The maximum extent of reduction by ascorbate is represented by spectrum ***8***, Fig. 1A. Subsequent addition of dithionite produces spectrum ***9***; the incremental change in absorbance is shown in Fig. 1B (***c***). This difference spectrum is distinct from that of spectrum of the low-potential heme center of cytochrome b_{561} being blue-shifted and having a larger separation between the peak and the shoulder (Table 1). Spectrum ***c*** accounts for approximately 15% of the absorption at

Table 1
Characteristics of the α-band observed in the difference absorption spectra of three heme centers of CGM

	Room temperature		Liquid nitrogen temperature	
	Peak (nm)	Shoulder (nm)	Peak (nm)	Shoulder (nm)
Low-potential heme center	562	557.6	559.8	554.9
High-potential heme center	560.5	554.6	557.8	551.4
Ascorbate non-reducible heme center	561.0	554.1	557.2	550

561 nm and we suggest that it represents another heme center, non-reducible by ascorbate, the presumed physiological reductant for cytochrome b_{561} [22]. The same amount of this species was found in all four preparations examined.

While the differences in the room temperature spectra are small and might be overlooked upon superficial examination, these differences are convincingly apparent when a similar experiment is carried out at liquid nitrogen temperatures. The spectra of individual heme centers, obtained from such an experiment, are shown in Fig. 2. As expected, lowering the temperature sharpens the spectra and a shoulder of the low-potential heme can be observed directly in spectra of CGM reduced by the highest concentrations of ascorbate (not shown) and in dithionite-reduced minus ferricyanide-oxidized samples (Fig. 2, ***1***). Spectra ***2***–***4*** of Fig. 2 represent individual heme centers of CGM; their parameters are summarized in Table 1. In full accord with the room temperature results the spectrum of the low-potential heme center of cytochrome b_{561} is narrower and red-shifted relative to the high-potential species. The spectrum of the ascorbate non-reducible heme center (Fig. 2, ***4***) resembles that of the high-potential heme (***2***) but its α-band is more split.

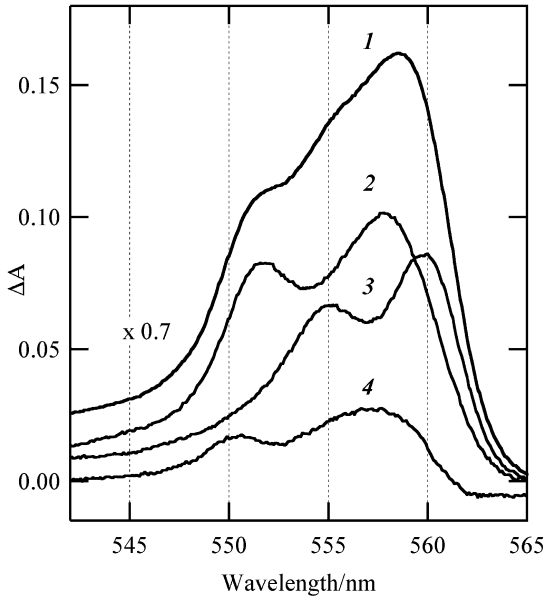

Fig. 2. Reduced minus oxidized difference absorption spectrum of CGM and individual difference spectra of CGM heme centers collected at 77 K. ***1***: Dithionite-reduced versus oxidized CGM; the sample was oxidized by 8 μM of ferricyanide (note that amplitude of this spectrum was scaled down by a factor of 0.7). ***2***: Spectrum of the high-potential heme center calculated as the difference between the spectra of CGM reduced by 1 mM and 300 μM ascorbate. ***3***: Spectrum of the low-potential heme center calculated as the difference between spectra of CGM reduced by 60 mM and 1 mM ascorbate. ***4***: Spectrum of the ascorbate non-reducible heme center calculated as the difference between spectra of CGM reduced by dithionite and 60 mM ascorbate. As was noted by Flatmark and Terland a small portion of cytochrome b_{561} cannot be reduced even by a high concentration of ascorbate [8]. According to our estimate approximately 80% of low-potential heme center was reduced by 60 mM of ascorbate in this experiment. Correspondingly, spectrum ***4*** was corrected by subtracting 20% of spectrum ***3***. The incubation media are the same as in Fig. 1.

Utilizing samples of CGM prepared according to traditional methods [24] instead of those adopted for this study we were unable to observe a distinct difference in the positions of the maxima of the low-potential and high-potential hemes, even in absorbance spectra recorded at liquid nitrogen. This we interpret as meaning that the low-potential heme is labile and is either partially lost during the long preparation or is modified and exhibits absorption spectra similar to that of high-potential heme, or both. Nevertheless, the optical properties of the low-potential heme were somewhat preserved in the traditional preparation [24] as well because the lineshape of the spectra of the two heme centers was different (not shown), as was reported previously [10]. The absorption spectra of the high-potential heme and the EPR signals of high- and low-potential heme centers were similar for both types of CGM preparations.

Kamensky et al. [10] and Burbaev et al. [11] have reported that the EPR spectrum of a fully oxidized sample of CGM exhibits two different types of low-spin heme EPR signals; a high-potential signal with g_z near 3.1 and a low-potential signal with $g_z = 3.70$ (EPR spectra at high level of reduction were not investigated). We have reproduced their results (Fig. 3, upper) and extended it by including a sample that was approximately 85% reduced with 60 mM of ascorbate (Fig. 3, lower). This sample also exhibited two signals, one with $g_z = 3.70$ that belongs to the remaining oxidized fraction of the low-potential heme of cytochrome b_{561} and a second, new low-spin signal located to slightly higher magnetic field; the high-potential center is fully reduced with this concentration of ascorbate and thus no EPR signal is present at $g_z = 3.1$ (not shown). At 11 K and 50 mW microwave power we measured the g_z of the new signal to be 3.62, a value typical of a well-organized HALS center and is thus unlikely to represent some denatured cytochrome b_{561}. In addition none of these samples exhibited a $g = 6$ high-spin EPR signal which might be expected to be generated upon denaturation (not shown). The possibility of mitochondrial contamination in the preparation of CGM can be excluded as no trace of absorbance at 600 nm characteristic of cytochrome oxidase was observed, nor were any EPR signals attributable to mitochondria detected in any of the samples.

4. Discussion

Historically all optical studies of membranous cytochrome b_{561} were conducted using membranes prepared by a fairly elaborate procedure involving several freeze-thawing cycles

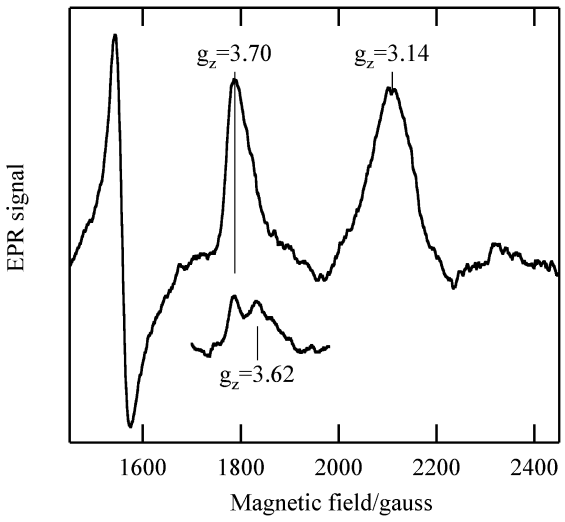

Fig. 3. EPR spectra of oxidized (upper) and 85% reduced (lower) CGM. Spectra were collected at 11 K. Other conditions: microwave frequency 9.273 GHz; modulation frequency 20 Gauss; microwave power 50 mW; time constant 1 s; the upper spectrum is a result of averaging four spectra while the lower one was averaged 20 times.

and osmotic shock to lyse CG [4] followed by 48 h dialysis to remove endogenous substrates interfering with the redox titration of the cytochrome [19], sometimes in combination with a high salt wash [24]. In this study we utilized the gentler procedure of Huyghe and Klinman [21], who tried to prepare more viable vesicles from CGM, by incorporating two key steps, namely the use of glycerol to lyse the CG [25,26] and Sephadex chromatography for rapid removal of substrates [20].

Apps et al. [6] first collected high quality optical spectra of membranous cytochrome b_{561} during a redox titration. Although their potentiometric data indicated that there were at least two hemes with different redox potentials they were unable to associate these hemes with specific spectral forms. It is apparent from our data (Figs. 1 and 2) that two clearly different spectral forms of cytochrome b_{561} exist. The spectra of the individual heme centers of CGM that we have obtained (Figs. 1B and 2) explain why the absolute absorption spectra of cytochrome b_{561} reported by Apps et al. (figure 2 of [6]) were visibly split during the early stages of reduction, but were much less asymmetric towards the end. The shoulder of the spectrum of the low-potential heme coincides fortuitously with trough in the spectrum of high-potential species so the latter becomes obscured as the titration progresses (cf. ***2*** and ***7***, Fig. 1A). At 77 K, a third maximum, near 555 nm, representing a shoulder in the spectrum of the low-potential species, is clearly present in the spectrum of CGM dithionite-reduced minus ferricyanide-oxidized (Fig. 2, ***1***).

The demonstration of independent spectra for the two heme centers in cytochrome b_{561} is of practical significance because it provides a simple optical tool for monitoring the two centers individually and provides further support for the existing models.

The finding of a third heme center in CGM was surprising. Its g_z value of 3.62 places it in the HALS class thus making it very unlikely to be a denatured species (the HALS signal requires an unusual orientation of the axial ligands that is lost when steric constraints are removed). Although we cannot exclude the possibility that this new species is a form of b_{561} that lacks the ability to react with its substrate, the possibility must be considered that this center is a distinct species; this new species would be a low-potential *b*-type cytochrome, most probably with bis-histidine coordination. It will be important to establish whether or not this component is present in purified preparations of cytochrome b_{561}.

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