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Several forms of chromaffin granule cytochrome *b*-561 revealed by EPR spectroscopy

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Low-temperature EPR spectra of chromaffin granule membranes from bovine adrenal medulla reveal 3 different signals of the ferric cytochrome *b*-561. A typical g_z signal of a low-spin cytochrome observed at $g \sim 3$ is comprised of a high-potential component with $g_z = 3.14$ and a low-potential one with $g_z = 3.11$, the low-potential signal showing significantly faster relaxation. In addition, a highly temperature-sensitive heme signal at $g = 3.7$ is observed which is fully retained in the preparation of granule membranes with *b*-561 reduced by 50% but disappears upon full reduction of the cytochrome by ascorbate. The signal is strikingly similar to that of the mitochondrial low-potential cytochrome *b* heme (b_L or *b*-566). The presence of several forms of *b*-561 in chromaffin granule membranes may provide a structural basis for the transmembrane electron transfer believed to be catalyzed by this hemoprotein.

Chromaffin granule; Cytochrome *b*-561; Electron paramagnetic resonance; Hemoprotein; Catecholamine

1. INTRODUCTION

Cytochrome *b*-561 is believed to play a key role in electron transfer across membranes of chromaffin granules required for noradrenaline synthesis inside these specialized organelles of the secretory cells of the adrenal medulla [1]. The mechanism of this process remains unknown. The cytochrome is a highly hydrophobic hemoprotein with a molecular mass of ca. 30 kDa and an amino acid sequence which shows no apparent homology with any other membrane-bound cytochrome *b* so far known and contains 6 typical transmembrane α-helixes [2]. The cytochrome is characterized by a rather high redox midpoint potential and an asymmetric absorption peak in the α-band with a maximum at 561 nm and a shoulder at approx. 558 nm [3,4].

In 1984, Abbs et al. [4] revealed the presence of two potentiometrically different forms of cytochrome *b*-561 and reported the high- and low-potential components to have identical absorption spectra. Redox heterogeneity of *b*-561 in chromaffin granule membranes was confirmed in this group [5], but in contrast to Abbs et al. [4] we found the high- and low-potential cytochrome to display a markedly different lineshape of their reduced *minus* oxidized difference spectra in the α-band [6].

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Abbreviation: CGM, chromaffin granule membranes

In this work we report on the EPR characteristics of cytochrome *b*-561 which have not been studied earlier to the best of our knowledge. The data indicate clearly the presence of multiple forms of the cytochrome in CGM.

2. MATERIALS AND METHODS

Chromaffin granule membranes were isolated from bovine adrenals according to [7] and dialysed for 2 days against a 100-fold volume of a buffer containing 10 mM MOPS, 0.1 mM EDTA, pH 7.2, with 5 changes of the buffer. After this treatment, about 5% of *b*-561 retained the reduced state due to endogenous substrates; these membranes were considered as 'oxidized'. The reduction level of *b*-561 was adjusted by adding different concentrations of ascorbate, while dithionite was used to ensure complete reduction and controlled in Aminco DW 2a or Hitachi 557 spetrophotometers. An appropriately reduced sample of CGM was transferred from a spectrophotometer cell (usually 2 mm optical pathway) to 4 mm inner diameter quartz tubes and frozen in liquid nitrogen. EPR spectra were recorded in a 'Radiopan SE/X 2544' spectrometer equipped with a custom-built cryostat for liquid helium temperature measurements.

3. RESULTS

Fig. 1A shows a typical EPR spectrum of freshly isolated and dialyzed CGM at 30K. Besides the signals of copper at $g = 2.06$ and high-spin non-heme iron at $g = 4.3$, the spectrum shows a well resolved $g_z = 3.12$ peak of a low-spin cytochrome which can be attributed to ferric cytochrome *b*-561. The central component of the cytochrome spectrum at $g \sim 2.14$ is overlapped by the copper signal and the g_x peak is too broad to be

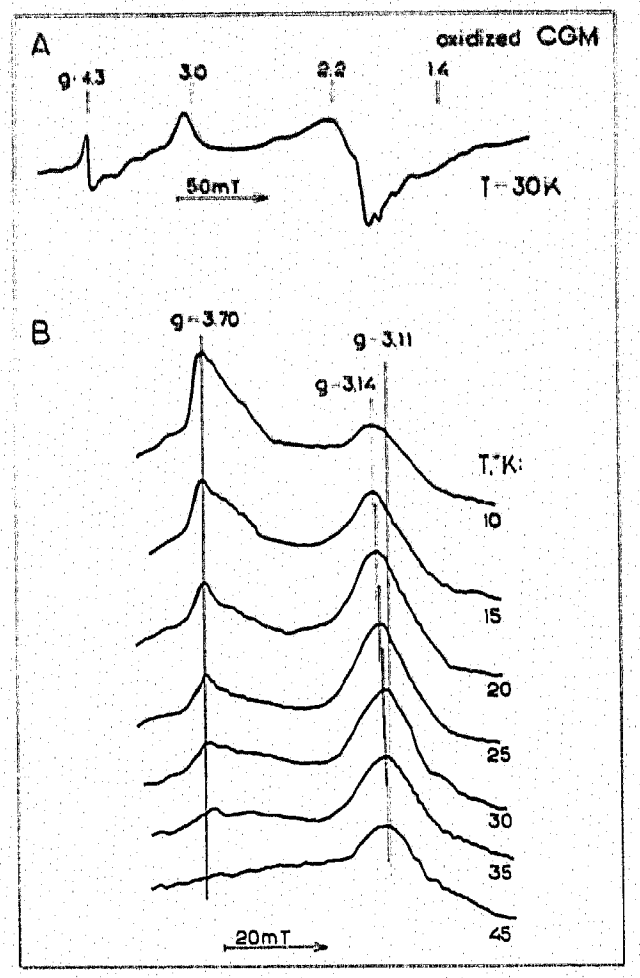

Fig. 1. Low temperature EPR characteristics of the oxidized chromaffin granule membranes. (A) An overall EPR spectrum of the oxidized CGM at 30K. (B) Temperature dependence of cytochrome *b*-561 g_z EPR signals in the oxidized CGM. The sample contained oxidized chromaffin granule membranes (cytochrome *b*-561 less than 5% reduced) at a concentration of 17 mg protein/ml in the medium containing 0.3 M sucrose, 30 mM MOPS, 0.1 mM EDTA, pH 7.2. EPR spectra were recorded at the indicated temperatures. Other conditions: microwave frequency, 9.304 GHz; modulation frequency, 100 kHz; modulation amplitude, 1 mT; microwave power, 50 mW; time constant, 1 s; scan rate, 75 mT/min. 16 spectra were averaged in each case.

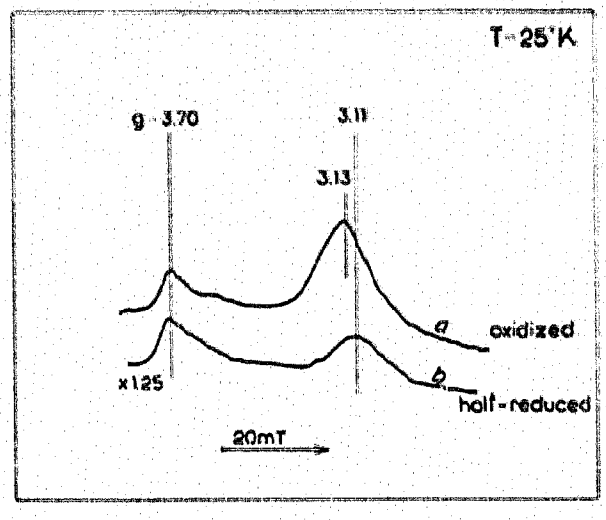

Fig. 2. EPR spectra of chromaffin granule membranes with cytochrome *b*-561 fully oxidized (a) and half-reduced (b). (a) Conditions as in Fig. 1. (b) The sample was adjusted with ascorbate to 50% reduction of *b*-561 as monitored spectrophotometrically in the α-band. T = 25K.

resolved accurately. Reduction by excess ascorbate or dithionite resulted in a complete disappearance of the cytochrome signals. Generally the EPR spectrum of *b*-561 is much like that of cytochrome b_5 from liver microsomes recorded under the same conditions (data not shown).

Fig. 1B shows the effects of temperature on the g_z signal of the cytochrome *b*-561 EPR signal. As the temperature is lowered from 45K to 10K, the height of the peak at $g \sim 3$ first increases, reaching a maximum at ~25K, and then decreases again; concomitantly, the peak position shifts from 3.11 to 3.14.

This effect indicates the $g \sim 3$ peak of cytochrome *b*-561 to be comprised of at least two components with slightly different g_z factors and significantly different relaxation characteristics, the higher field component saturating more easily.

In addition to the effect on the $g \sim 3$ peak, lowering the temperature reveals an additional highly asymmetric EPR signal with $g = 3.70$ which is strikingly similar to the g_z signal of the mitochondrial [8–10] and bacterial chromatophore [11] low-potential cytochrome *b* heme (b_L or *b*-566).

It was of obvious interest to relate the different EPR signals of cytochrome *b*-561 to the high- and low-potential forms of the cytochrome observed in redox titrations with the use of optical absorption spectroscopy [4,5]. As shown in Fig. 2, reduction of CGM cytochrome *b*-561 by 50% results in a significant decrease of the $g \sim 3$ peak height (about 3-fold at 25K) with a concomitant shift of the peak position to higher field similar to the shift observed upon raising the temperature (cf. Fig. 1B). On the other hand, the $g = 3.70$ signal is not affected appreciably.

These data indicate that the high-potential form of *b*-561 corresponds to the rapidly relaxing component with $g_z = 3.14$, whereas the low potential species is comprised of the more slowly relaxing heme with $g = 3.11$ and an additional heme centre with the unusual *b*-566-like EPR signal with $g = 3.70$, which is extremely temperature-sensitive.

Complete resolution and quantification of the 3 signals based on a detailed analysis of their temperature dependence will be described elsewhere. Preliminary estimates indicate the 3 components to be present in a roughly 1:1:1 stoichiometry.

4. DISCUSSION

Cytochrome *b*-561 is believed to carry out the electron transfer across the chromaffin granule membrane required for the regeneration of reduced ascorbate inside the granules [1,12].

Apparently, the idea of the transmembrane electron transfer has been borrowed from the 'redox loop' concept of Mitchell developed with respect to the mitochondrial and photosynthetic redox chains [13,14]. Although vectorial electrogenic electron transfer steps have been identified in the case of bacterial photosynthetic reaction centres [14–16] and are very likely to be inherent to the cytochrome complexes of the respiratory chain (see [18] for review), in neither case the transmembrane charge separation is catalyzed by a single redox centre-containing protein.

Accordingly, it is not easy to visualize transmembrane electron flow mediated by cytochrome *b*-561, the latter being a single-heme protein homogeneously oriented in the membrane. A possibility of *b*-561 being a two-heme protein analogous to the mitochondrial cytochrome *b* was considered recently [19].

The present data clearly show that viewing cytochrome *b*-561 as a single cytochrome species in CGM is probably an oversimplification. The presence of multiple forms of cytochrome *b*-561 heme revealed in CGM by redox titrations [4,5], optical absorption [6], and now by EPR spectroscopy, may provide a clue towards the understanding of the cytochrome operation as the transmembrane electron carrier in these organelles and questions the validity of the molecular structure of this hemoprotein suggested in [2]. This structure, assuming histidine-113 and methionine-52 as the axial ligands of cytochrome *b*-561 heme iron, is also in conflict with the optical and magnetic circular dichroism spectral characteristics of *b*-561 which point to His–His ligation of the heme iron and definitely exclude methionine coordination to the heme iron [20].

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