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CYTOCHROME b_{561} OF THE BOVINE ADRENAL CHROMAFFIN GRANULES

A HIGH POTENTIAL *b*-TYPE CYTOCHROME

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SUMMARY

Simultaneous potentiometric and spectrophotometric assays of cytochrome b_{561} oxidation–reduction in chromaffin granule membrane preparations (depleted of catecholamines and ATP) have been carried out under strictly anaerobic conditions. The cytochrome oxidation–reduction is described by a one-electron transition with an apparent midpoint potential at pH 7.0 (E'_{m_7}) of +140 mV. This value is in good agreement with the rate and extent of its aerobic reduction by ascorbate; the K'_m for the reductant was found to be 0.34 mM. The available evidence therefore confirms our previous suggestion that cytochrome b_{561} is a unique mammalian *b*-type cytochrome of chromaffin tissues.

INTRODUCTION

Our recent studies on chromaffin granule membrane preparations of bovine adrenal medulla[1] as well as of sympathetic nerve trunk[2] have revealed the presence of a unique electron transport system in these organelles including at least two types of electron carriers in addition to dopamine β-hydroxylase, *i.e.* flavoprotein(s) and a unique *b*-type cytochrome (termed b_{561}). Recent experiments also indicate that the two types of electron carriers, which are integral parts of the granule membrane structure, probably are linked to the main biosynthetic function of these organelles *i.e.* the formation of noradrenaline[3]. These observations have stimulated our efforts to achieve a more detailed physicochemical characterization of the individual electron carriers, particularly the flavoprotein(s) and the cytochrome b_{561}.

In the present study the standard oxidation–reduction potential of membrane-bound cytochrome b_{561} has been determined in order to establish its possible position in the electron transfer chain of the chromaffin granule membrane.

MATERIALS AND METHODS

Chromaffin granule preparations

The chromaffin granules were isolated from fresh bovine adrenals using a slight modification[4] of the procedure described by SMITH AND WINKLER[5]; the pellet P_c was used. The chromaffin granules, obtained by sedimentation in 1.6 M sucrose,

were given osmotic shock and homogenized to release their soluble contents. The granules were carefully resuspended in ice-cold 50 mM potassium phosphate buffer of pH 6.8 (approx. 1 ml/40 mg of protein) by homogenization (manually) in a Teflon-glass Potter–Elvehjem-type homogenizer. The homogenate, containing lysed granules and the released material together with some intact granules, was subjected to extensive dialysis[6] at 4° against approx. 500 vol. of phosphate buffer. Visking dialysis tubing retaining bovine cytochrome *c* (mol. wt. 12 130) quantitatively, was used. The dialysate was changed 8 times at 3-h intervals. Assuming equilibrium was attained each time, more than 99 % of any freely dialyzable substance should have been removed. The final retentate was resuspended by careful homogenization (see above) before use.

Reagents

Deionized water was used in making up all solutions. Analytical grade salts were used without purification. Sodium dithionite solutions were prepared by adding (under a nitrogen atmosphere) 0.5 g of sodium dithionite (Merck AG, Germany) to 50 ml of the deoxygenated buffer used in the titration experiments. After the dithionite dissolved, the solution was sucked into a syringe previously freed from oxygen.

Oxidation–reduction potentials

Oxidation–reduction titrations of cytochrome b_{561} were carried out essentially as described by DUTTON[7] and DUTTON *et al.*[8]. The equipment employed enabled simultaneous assay of oxidation – reduction potential (combined platinum and calomel electrode from Radiometer, Copenhagen, Model PK 149) and absorbance changes (Aminco-Chance dual-wavelength spectrophotometer) under strictly anaerobic conditions. Anaerobic conditions as well as stirring were effected during titration by maintaining a slow flow of purified nitrogen through the solution. A minimum amount of Antifoam A was added to avoid foaming. A freshly prepared solution of sodium dithionite in anaerobic buffer was used to establish potentials lower than +100 mV and oxidation–reduction potentials were made more positive with 0.1 M potassium ferricyanide in anaerobic buffer. An electron tube potentiometer (Radiometer, type Digital PHM 52) was used for registering the potentials. When equilibrium potentials were reached, the absorbance difference between $\lambda_2 = 561$ nm and $\lambda_1 = 550$ nm was recorded[1]. The determinations were carried out at a temperature of 25 ± 1°.

Spectrophotometry

The changes in the oxidation–reduction state of membrane bound cytochrome b_{561} was followed in cuvettes of 10-mm light path using an Aminco-Chance dual-wavelength spectrophotometer; the reference wavelength was set at 550 nm and the measuring wavelength set at 561 nm[1].

RESULTS AND DISCUSSION

Because chromaffin granules contain a large amount of endogenous oxidant/reductant, *i.e.* the catecholamines, it was important to remove these amines as

quantitatively as possible by extensive dialysis (see MATERIALS AND METHODS). When the extent of cytochrome b_{561} oxidation–reduction in the final retentate was measured in the dual-wavelength spectrophotometer, it was found to be present mainly in the oxidized form. Upon dilution of the sample in the cuvette, however, complete oxidation was effected within 10 min.

It has previously been found[1] that membrane-bound cytochrome b_{561} is reduced very rapidly by sodium dithionite and also readily by ascorbate. From Fig. 1 it is seen that about 85 % reduction occurred in less than 1 min at an ascorbate concentration of 9.6 mM. From the data given in Fig. 2 the K'_m for ascorbate was found to be 0.34 mM. For the purpose of comparison it should be mentioned that solubilized microsomal cytochrome b_5 ($E_{m7} = +20$ mV, see below) is reduced only to the extent of approx. 15 %[9] under the same experimental conditions with a K'_m value of 12 mM for ascorbate[10]. This fact suggests that the oxidation–reduction potential of cytochrome b_{561} is far above + 20 mV, and this suggestion was confirmed by the exact determination of this potential *in situ*, *i.e.* the cytochrome being an integral part of the granule membrane structure.

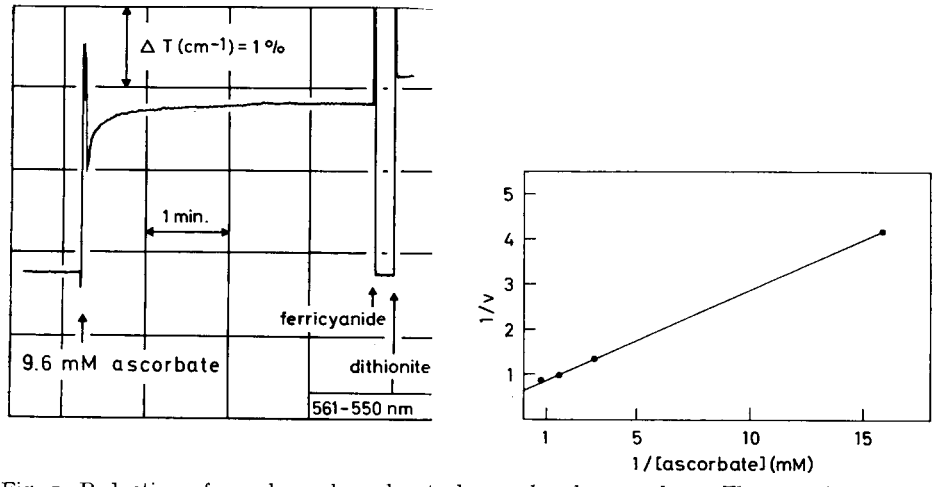

Fig. 1. Reduction of membrane-bound cytochrome b_{561} by ascorbate. The cuvette contained: 50 mM potassium phosphate buffer, pH 6.5; membrane preparation, 1.30 mg of protein per ml; 25°. The rate of formation of reduced cytochrome b_{561} was measured with an Aminco-Chance dual wavelength spectrophotometer.

Fig. 2. Effect of ascorbate concentration on the rate of cytochrome b_{561} reduction when plotted in a double reciprocal manner. Assay conditions were as described in Fig. 1, except that the ascorbate concentration was varied as shown. The rate (v) was expressed as the percentage decrease in transmission during the first 5 sec (ΔT (%) = $(T_{561\text{ nm}} - T_{550\text{ nm}})/5$ sec).

Various redox buffers were tested in the preliminary experiments, but the buffer 5 mM EDTA – 50 μM $FeCl_3$ was finally selected due to its favourable midpoint potential ($E_{m7} = +120$ mV)[11]. Fig. 3A describes two representative oxidative titrations of cytochrome b_{561} in a freshly prepared membrane preparation of adrenal chromaffin granules. Fitting the Nernst equation to the experimental values results in the solid curve shown in Fig. 3A. It is seen that the oxidation–reduction is best described by a one-electron transition. However, when the data were presented

graphically as the logarithm of the ratio Ox/Red as a function of the electrode potential (Fig. 3B) a slight sigmoidal curve was obtained. This small deviation from linearity was quite reproducible and may point to the existence under the experimental conditions used of two different forms of this electron carrier with midpoint potentials very close to each other. Thus, it is well known that the oxidation–reduction potential of *b*-type cytochrome in mitochondria and microsomes as well as in bacteria is very sensitive to environment[12–15]. Therefore, the mean and apparent midpoint potential of cytochrome b_{561} is given from the solid line of Fig. 3B, *i.e.* $E'_{m7} = +140$ mV, and the cytochrome should thus be classified as a high potential *b*-type cytochrome.

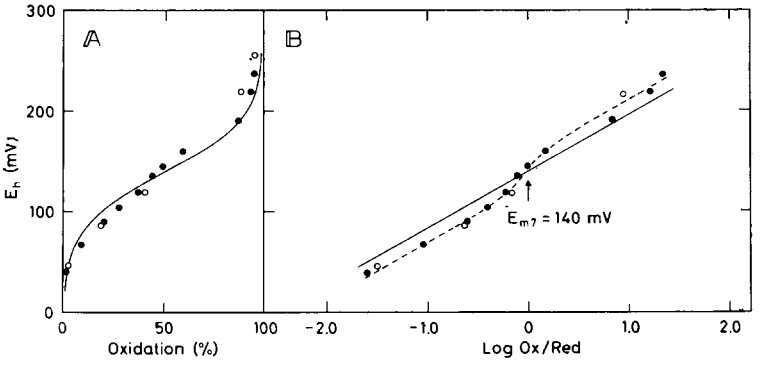

Fig. 3. Oxidation–reduction titrations of cytochrome b_{561}. The points (● and ○) are the experimental values of two separate titrations of a single preparation of chromaffin granules (1.4 mg of protein per ml). The solid lines represent the theoretical curves for $n = 1$; $E'_{m7} = +140$ mV. Oxidative titration was performed with potassium ferricyanide in 50 mM phosphate buffer (pH 7.0) at 25°; for details, see text.

The midpoint potentials of previously reported mammalian *b*-type cytochromes are all lower than that found for cytochrome b_{561} in the present study. Thus, E_{m7} for cytochrome b_5 of bovine liver microsomes[14–16] is — 140 mV and + 20 mV for the membrane-bound and the solubilized form, respectively, and the major component of cytochrome *b* of bovine heart mitochondria (accounting for 55 % of the total *b*-type cytochrome) is +38 mV[8]. Minor contributions to the total spectral change of total *b*-type cytochrome of freshly prepared mitochondria is attributed to two components with $E_{m7.2}$ values of — 130 mV (24 %) and + 125 mV (21 %), respectively[8]. Thus, our present determination of oxidation–reduction potential of cytochrome b_{561} and the kinetics of its aerobic reduction by ascorbate confirms our previous suggestion that it is a unique member among the mammalian *b*-type cytochromes, characteristic of chromaffin tissues[1–2]. The elucidation of the oxidation–reduction potential of this cytochrome will now enable more detailed studies to be performed on its function.

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