Interaction of antimycin with cytochrome *b*-561

A study in secretory granules and in plasma membrane isolated from chromaffin cells of bovine adrenal medulla

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Received 4 July 1983

Cytochrome *b*-561 in chromaffin granules interacts with antimycin and its α-peak shifts 1 nm towards red. When chromaffin granules were treated with Triton X-100 antimycin no effect was observed. Cytochrome *b*-561 is located in the plasma membrane isolated from the chromaffin cells. The plasma membrane *b*-561 does not seem to interact with antimycin. A number of NADH or NADPH (acceptor) oxidoreductase activity has been observed in isolated plasma membrane providing clues to the origin of plasma membrane dehydrogenase. The possible role of cytochrome *b*-561 in secretory granules other than its accredited energy conserving electron transport property is projected.

Cytochrome b-*561* *Secretory granule* *Chromaffin granule* *Plasma membrane* *Dehydrogenase* *Antimycin* *Spectral shift*

1. INTRODUCTION

Cytochrome *b*-561 is present in chromaffin granules [1], serotonin-dense granules [2] and in noradrenaline storage vesicles [3]. The physiological role of cytochrome *b*-561 in these organelles remains obscured although its function in mitochondria is well-founded. In spite of best efforts by several groups [4–6] no meaningful role, in terms of physiological function of chromaffin granules, has been assigned to cytochrome *b*-561, mainly due to its location; i.e., facing the cytosolic exterior [7]. However, it is known that in chromaffin granules cytochrome *b*-561 can be oxidized by ferricyanide, can be reduced by ascorbate [8] and has a midpoint potential of +140 mV at pH 7.0 [9]. Furthermore, cytochrome *b*-561 in chromaffin granules does not accept electrons from NADH and its endogenous oxidase remains to be identified. In isolated chromaffin cell plasma membrane, cytochrome *b*-561 activity and its reducibility with NADH was identified together with latent NADH–cytochrome *b*-561 reductase activity (A.N.M., T. Flatmark, in preparation). Here, we confirm the location of cytochrome *b*-561 in chromaffin cell plasma membrane and present evidence for the occurrence of a number of NADH/NADPH (acceptor) oxidoreductases.

The uniqueness of cytochrome *b*-561 in mitochondria [10] is its interaction with antimycin causing a spectral shift towards red. We report that cytochrome *b*-561 in chromaffin granules interacts with antimycin and an obvious spectral shift occurs. But with cytochrome *b*-561 in plasma membrane one does not see the antimycin effect. The possible function of cytochrome *b*-561 in secretory organelles other than its energy-conserving electron transport property is postulated.

2. EXPERIMENTAL

The chromaffin granules from bovine adrenal

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

medulla were prepared according to [11] as modified in [12]. The plasma membrane from the chromaffin cells were isolated essentially as in [13], except that in the final step plasma membrane fraction was collected at the interphase of 1.0–1.2 M sucrose in a discontinuous gradient after centrifugation at 150000 × *g* for 14 h. NADH or NADPH (acceptor) oxidoreductase activity was measured as in [14]. Cytochrome *c* oxidase assay was done as recommended in [15]. Difference spectra (reduced minus oxidized) were recorded utilizing Aminco DW-2 and Beckman Acta III spectrophotometers. The protein was determined as in [16] employing bovine serum albumin as standard. All enzyme assay and difference spectroscopic studies were performed in thermostated cuvettes at 30°C. Cytochrome *c* (horse heart type VI), glyoxylate, antimycin A, dichloroindophenol, NADH/NADPH (each in 1 mg pre-weighed vials) and rotenone were from Sigma. Other reagents were of analytical grade.

3. RESULTS

Fig.1. illustrates the interaction between cytochrome *b*-561 and antimycin A in chromaffin granules. The difference spectrum, ascorbate reduced minus oxidized was recorded (trace B), the addition of antimycin (trace C) induced a shift of the α-peak from 561 nm to 562 nm; when ascorbate was added to the reference cuvette (trace D) the resultant spectrum of antimycin-shifted *b* species with the α-peak at 562 nm was seen. Fig.2 illustrates the antimycin effect on cytochrome *b*-561 in chromaffin granules treated with Triton X-100 (trace B reduced minus oxidized). The addition of antimycin to the sample cuvette (trace C) did not shift the α-peak. Fig.3 shows the difference spectra of cytochrome *b*-561 in isolated plasma membrane: trace B is the ascorbate reduced minus oxidized spectrum; and the addition of antimycin leaves cytochrome *b*-561 unaffected (trace C). Likewise, when plasma membrane was treated with Triton X-100 no spectral shift due to antimycin was observed (not shown) indicating non-interaction of plasma membrane *b*-561 with antimycin.

The extent of reduction of cytochrome *b*-561 in chromaffin granules as compared to the plasma membrane is illustrated in table 1. In chromaffin

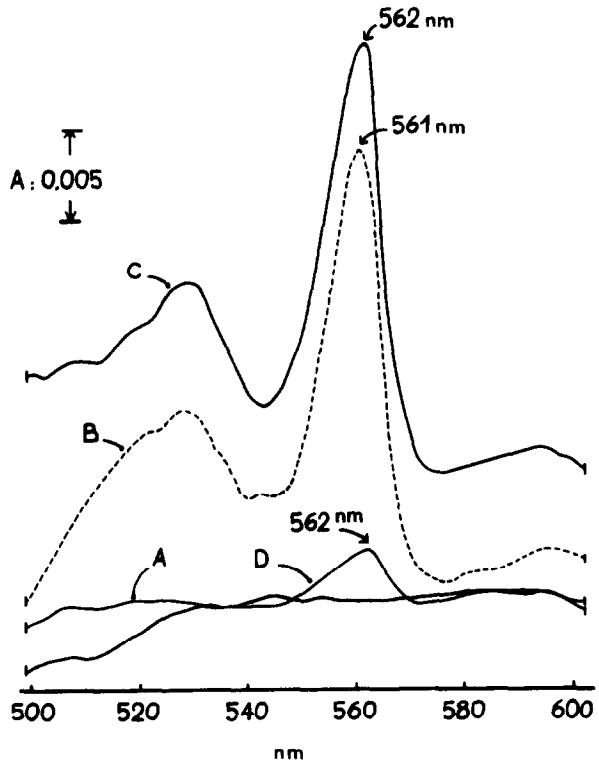

Fig.1. Difference spectra of chromaffin granules: 50 mM phosphate buffer (pH 7.0) reduced by ascorbate (10 mM) and oxidized by ferricyanide (10 μM); (A) oxidized/oxidized; (B) reduced/oxidized; (C) reduced + antimycin (5 μM)/oxidized showing spectral shift of 1 nm in cytochrome *b*-561; (D) ascorbate + antimycin/ascorbate. Antimycin was dissolved in 95% ethanol and an equal amount of ethanol was added to the reference cuvette in the case of (C). Baseline corrections were made. Chromaffin granules were present in 1.16 mg protein/ml.

granules, about 93% cytochrome was reduced by ascorbate taking dithionite reduction as 100%. While in plasma membrane slightly lower reduction was obtained. The two membranes were influenced differently with respect to *b*-561 reducibility in the Triton-treated system. In chromaffin granules Triton treatment did not enhance ascorbate reducibility while the reduction by dithionite was slightly decreased. This may be attributed to the non-specific perturbation of chromaffin granules caused by the detergent or the solubilization of some component of secretory system interacting with dithionite and interfering

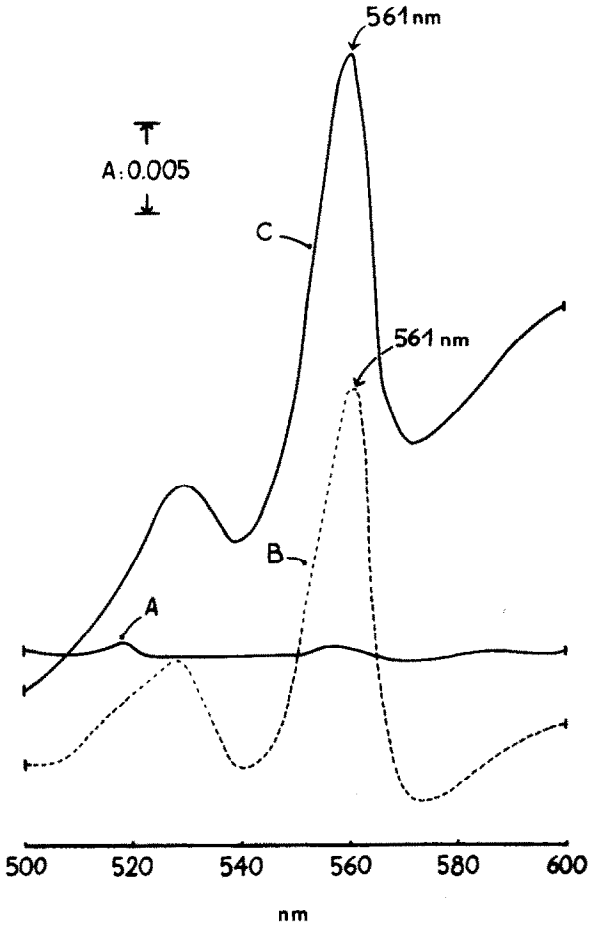

Fig.2. Difference spectra of chromaffin granules. The experimental conditions were the same as in fig.1, except that the chromaffin granules were treated with Triton X-100 (1%): (A) oxidized/oxidized; (B) ascorbate/oxidized; (C) ascorbate + antimycin/oxidized. Chromaffin granules were 1.05 mg protein/ml.

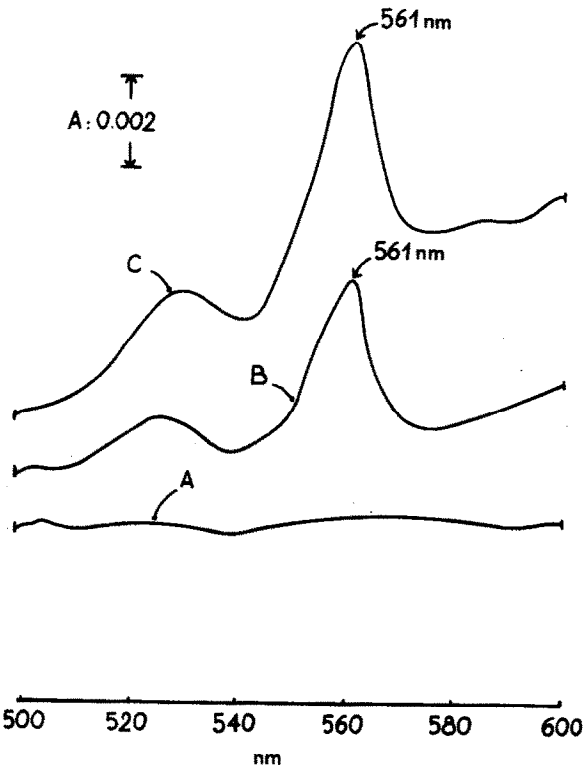

Fig.3. Difference spectra of plasma membrane. The experimental conditions were the same as in fig.1: (A) oxidized/oxidized; (B) ascorbate/oxidized; (C) ascorbate + antimycin/oxidized. Plasma membranes were present in 1.22 mg protein/ml.

spectrally. On the contrary, there was nearly a 2-fold increase in the reduction and specific content of cytochrome *b*-561 in the plasma membrane upon Triton treatment both with ascorbate or dithionite employed as reductant. A ratio ranging from 0.14–0.39 of plasma membrane:chromaffin granules *b*-561 specific content suggests the actual localization of this protein in the plasma membrane and not an artefact of preparation.

Table 2 summarizes various acceptor oxidoreductase activities in 3 representative preparations of plasma membrane. There was no detectable cytochrome *c* oxidase activity present and neither NADPH cytochrome *c* reductase nor rotenone-sensitive NADH cytochrome *c* reductase activity were found. The NADH dehydrogenase system responsible for reduction of ferricyanide or dichloroindophenol showed large activities as compared to NADPH-linked dehydrogenase, with the exception of dehydrogenase catalyzing glyoxylate reduction. The ratio of NADH-ferricyanide reductase:NADH cytochrome *c* reductase activity was 4.0, according to the data in table 2 (a mean value of the 3 preparations), while in chromaffin granules this ratio was 0.4 [17], indicating that this is plasma membrane dehydrogenase.

Table 1

Specific activity of cytochrome *b*-561 in chromaffin granules (CG) as compared with the plasma membrane (PM)

Reductant	CG (nmol/mg protein)	PM (nmol/mg protein)	PM / CG
Ascorbate	0.088	0.013	0.147
Ascorbate + 1% Triton X-100	0.084	0.025	0.297
Dithionite	0.095	0.015	0.157
Dithionite + 1% Triton X-100	0.082	0.032	0.390

These values are based on reduced minus oxidized difference spectra employing 26.8 $mM^{-1}.cm^{-1}$ as molar extinction coefficients at the wavelength pair 561–575 nm [18]. The membrane preparation was oxidized with ferricyanide (10 μM), and ascorbate was 10 mM

Table 2

Acceptor oxidoreductase activity in plasma membrane of chromaffin cells

Acceptor oxidoreductase	Spec. act. (nmol.min^{-1}.mg $protein^{-1}$)		
	Prep.(a)	Prep.(b)	Prep.(c)
NADH ⟶ cytochrome *c*	26.8	26.1	32.0
NADPH ⟶ cytochrome *c*	nil	nil	nil
NADH ⟶ glyoxylate	8.4	7.1	9.1
NADPH ⟶ glyoxylate	8.4	15.9	9.1
NADH ⟶ ferricyanide	132.9	124.5	81.3
NADPH ⟶ ferricyanide	12.9	8.6	8.1
NADH ⟶ dichloroindophenol	34.0	35.0	27.9
NADPH ⟶ dichloroindophenol	4.0	3.6	4.5

50 mM phosphate buffer (pH 7.0), NADH (100 μM), NADPH (100 μM), cytochrome *c* (50 μM), glyoxylate (4 mM), ferricyanide (100 μM), dichloroindophenol (100 μM). In cytochrome *c* reductase assay 3 mM sodium cyanide was present. The total volume of the reaction mixture was 1.0 ml. NADH–cytochrome *c* reductase activity was insensitive to rotenone. Preparation (a), (b) and (c) contained 30.7, 32.5 and 28.5 μg protein/ml in the reaction cuvette, respectively

4. DISCUSSION

Cytochrome *b*-561 in chromaffin granules faces cytoplasmic exterior and is ascorbate reducible in situ. It does not interact with internal cytochrome *c*, nor does it accept electrons donated by NADH in chromaffin granules [5]. It seems that in secretory granules it has one side of reduction. As shown in table 1, ascorbate reduced almost all the moiety, and upon Triton treatment no reducible *b*-561 component was rendered available. On the contrary, in plasma membrane the orientation of cytochrome *b*-561 appears to be markedly different from that in chromaffin granules. The 2-fold rise in specific content of cytochrome *b*-561 due to Triton treatment supports this statement. Furthermore, the reducibility of *b*-561 by NADH and the recognition of latent NADH cytochrome *b*-561 reductase activity (in preparation) indicate more than a single side of reduction.

In secretory granules cytochrome *b*-561 interacts with antimycin and its α-peak shifts towards red by 1 nm. Moreover, when chromaffin granules were treated with 1% Triton X-100 cytochrome *b*-561 does not see antimycin effect. Similarly in plasma membrane cytochrome *b*-561 does not interact with antimycin either in intact membrane, or membrane treated with 1% Triton X-100. Thus, it appears that the reactivity of antimycin with a redox protein like *b*-561 is solely due to the latter's orientation in the membrane as envisaged by the chemiosmosis concept. These findings are contrary to the observation in [18] where antimycin effects on *b*-561 in chromaffin granules were not seen. Furthermore, non-reduction of *b*-561 in chromaffin granules by durohydroquinone [18] is a weak criterion for distinction between chromaffin granules *vs* mitochondrial cytochrome, since the endogenous oxidase system for chromaffin granules *b*-561 remains unknown. It is evident here that cytochrome *b*-561 in chromaffin granules shares the property of mitochondrial *b*-561

towards its interaction with antimycin. Myxathiozol (M), another site II inhibitor of mitochondrial electron transport blocking the antimycin-insensitive site in mitochondria, does not interact with mitochondrial *b*-561. Likewise, *b*-561 in chromaffin granules does not show a myxathiozol effect [20]. Thus, *b*-561 in its native membrane orientation shares similar inhibitor effects in chromaffin granules and in mitochondria. This is the first report showing the presence of cytochrome *b*-561 in chromaffin cell plasma membrane although plasma membrane from neutrophils [22] and milk fat globules [23] were shown to contain the *b*-type cytochrome. But the detailed knowledge of *b*-561 in plasma membrane as compared to chromaffin granules will have to await future experimentation. Commenting on the mitochondrial *b*-561 *vs* *b*-561 in chromaffin granules, there is not sufficient detailed structural information on granule *b*-561 to establish any intrinsic difference or similarity between the two proteins (cf. [18]). Nevertheless, it may be argued that *b*-561 in chromaffin granules does not seem to participate in the actual physiological function of this organelle, while in mitochondria it has a genuine role in electron transport coupled to proton translocation.

What function may be assigned to a redox protein like *b*-561 other than energy-conserving electron transport in secretory granules or in plasma membrane? The following possibilities are worthy of consideration:

(i) Oxidoreduction causing conformational change in the protein affecting its binding to a receptor, or membrane permeability;
(ii) Transmembrane oxidoreduction (can *b*-561 species be attacked on more than one side of the membrane?);
(iii) Alterations in membrane-fixed charge, both by the redox changes and by Bohr effect;
(iv) Acting as or fuelling redox-linked pumps (not necessarily potential-generating proton pumps).

NADH or NADPH dehydrogenases are intrinsic plasma membrane dehydrogenases [17]. However, their origin in plasma membrane remains less known than the widely documented mitochondrial or microsomal dehydrogenases. Here a systematic study is reported on NADH/NADPH dehydrogenase in chromaffin cell plasma membrane employing a natural acceptor such as cytochrome *c*, a metabolic acceptor such as glyoxylate and an artificial acceptor such as ferricyanide or dichloroindophenol (table 2). Since no cytochrome *c* oxidase activity was detected, it is safe to conclude that the plasma membrane preparation was not contaminated by mitochondria. This is further supported by the observed rotenone-insensitive NADH cytochrome *c* reductase activity. The absence of NADPH cytochrome *c* reductase activity does exclude the possibility of microsomal contamination.

ACKNOWLEDGEMENTS

We are grateful to Dr J. Magdalov, Faculté de Pharmacie, Nancy, for the use of Aminco DW-2 spectrophotometer. A.N.M. acknowledges the financial support from the Ministère de la Recherche et de l'Industrie (Paris). We thank Mrs F. Herth for typing this manuscript.

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