
CHAPTER 6

The ascorbate:AFR oxidoreductase from the erythrocyte membrane is not cytochrome b_{561}

This chapter was modified from MM VanDuijn, J Van der Zee, and PJA Van den Broek, The ascorbate:AFR oxidoreductase from the erythrocyte membrane is not cytochrome b_{561} . *Protoplasma*, *In press*.

Summary

Erythrocytes contain a plasma membrane redox system that can reduce extracellular ascorbate radicals using intracellular ascorbate as an electron donor. In this study, the hypothesis was tested that cytochrome b_{561} was a component of this system. Spectroscopic analysis of erythrocyte membrane preparations revealed the presence of cytochrome b_5 and hemoglobin, but also of a cytochrome with properties similar to cytochrome b_{561} , reducible by ascorbate, and insensitive to CO. The presence of cytochrome b_{561} was studied further by RT-PCR analysis of erythrocyte progenitor cells, reticulocytes. However, no cytochrome b_{561} mRNA could be found. These results were corroborated by Western blot analysis with an anti-cytochrome b_{561} serum. No cytochrome b_{561} protein could be detected in extracts of erythrocyte membranes. It is therefore concluded that erythrocytes do not contain cytochrome b_{561} in their membranes. The possible involvement of other b -cytochromes in ascorbate-AFR oxido-reductase activity is discussed.

Introduction

Most cells contain a wide variety of systems to maintain their levels of anti-oxidants, such as ascorbate (vitamin C). These systems are especially important for primates and guinea pigs, which lack the capability for *de novo* synthesis of ascorbate. These species therefore rely on dietary intake of the vitamin and regeneration of oxidized ascorbate. Oxidation products of ascorbate include the ascorbate free radical (AFR) and dehydroascorbic acid (DHA). AFR is a relatively stable radical that is generated by the one-electron oxidation of ascorbate. In a second one-electron step AFR can be further oxidized to DHA. Alternatively, two molecules of AFR can disproportionate to form one ascorbate and one DHA molecule. Both AFR and DHA can be reduced back to ascorbate, but the fast and irreversible hydrolysis of DHA, with a half-life at 37 °C of approximately 10 min, asks for swift and efficient regeneration systems (1, 2).

Intracellularly, a number of reducing systems are available, such as glutathione (GSH), thioredoxin reductase, glutaredoxin, protein disulfide isomerase and a mitochondrial NADH:AFR reductase (3-9). For the reduction of extracellular oxidation products of ascorbate, cells can transport DHA *via* the GLUT-1 glucose transporter for intracellular reduction to ascorbate (2, 10, 11). Also, a transmembrane NADH:AFR reductase has been described for the extracellular reduction of AFR, while another group reported the extracellular reduction of DHA (12, 13). Recently, our group identified a new pathway in the erythrocyte for the reduction of

extracellular AFR (14). It was found that, similar to NADH, intracellular ascorbate can serve as the electron donor for the reduction of extracellular AFR.

While there is good evidence for ascorbate-dependent electron transport across the membrane, it is still not clear what the functional components of this system are. The transfer of electrons from ascorbate to ascorbate radicals, over a membrane, is not a common process. Only one protein, most commonly found in chromaffin granules, has been shown to be involved in such a process. This protein, cytochrome b_{561} , has been isolated and the cDNA sequenced in a number of species, including humans (15). Its structure seems to be rather unique, because no homologues of the protein are known in genomic databases, other than the cytochrome b_{561} from different species. Cytochrome b_{561} is highly expressed in the membrane of granules inside chromaffin cells of the adrenal medulla. In these granules, synthesis of catecholamines by dopamine- β -hydroxylase oxidizes large amounts of ascorbate to AFR. Cytochrome b_{561} transfers electrons from cytoplasmic ascorbate to reduce the AFR in the granules back to ascorbate (16). Similar *b*-cytochromes have been identified in the plasma membrane of the plant *Phaseolus vulgaris*. They can reduce AFR and other substrates in the apoplast, using cytoplasmic ascorbate as an electron donor (17-19). Also, sequences have been identified in *Arabidopsis thaliana* with high homology to cytochrome b_{561} (e.g. Genbank #AF132115).

As cytochrome b_{561} has been found in several tissues and species, and as no other proteins have been described to transfer electrons from ascorbate to AFR, we hypothesized that cytochrome b_{561} might also be responsible for the transfer of electrons over the erythrocyte membrane. To investigate this, various techniques were used to gather evidence for the expression of cytochrome b_{561} in erythrocytes, and for its possible role in the reduction of extracellular ascorbate radicals.

Materials and Methods

Reticulocyte Isolation - Reticulocytes were isolated from EDTA anti-coagulated blood, freshly drawn by venipuncture from healthy human volunteers. Leukocytes were removed by filtration over a cellulose column with a bed of at least 1 cm (20). After elution from the column, erythrocytes were washed twice in sterile PBS, and once in PBS containing 0.1% BSA. Subsequently, 600 ml packed erythrocytes were mixed with 400 ml PBS/0.1% BSA and $8 \cdot 10^6$ Dynabeads M-450 coated with anti-CD71 antibody (Dynal, Oslo, Norway), and incubated for 45 minutes on a rolling mixer. After incubation, the beads were sedimented with a magnet,

and the pellet was rinsed with PBS/0.1% BSA until the supernatant remained clear. The beads were resuspended in PBS/0.1% BSA, and the procedure was repeated once. Analysis of the resulting pellet by staining with 0.5% New Methylene Blue in PBS showed that more than 99% of the remaining cells were reticulocytes.

Isolation of neutrophils - Human neutrophils were isolated from a buffy coat, obtained from the Bloodbank Leiden-Haaglanden, by starch sedimentation and Ficoll centrifugation (21). The neutrophils were suspended in 140 mM NaCl, 20 mM HEPES, 5 mM KCl and 10 mM glucose at pH 7.3.

K562 cells - K562 erythroleukemic cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 100 U/ml penicillin G, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 3 mM L-glutamine. Culture flasks were kept in a humidified atmosphere with 5% CO₂ at 37 °C. Cells were harvested when the culture had reached a density of $\sim 7 \cdot 10^5$ cells/ml, and were washed twice in phosphate buffered saline before use.

RNA isolation and cDNA synthesis - All (RT)-PCR reagents were from Promega (Leiden, The Netherlands) unless stated otherwise. Total RNA was isolated from cells utilizing RNeasy B (Qiagen Scientific, Venendaal, The Netherlands), according to the manufacturer's specifications. The RNA was found to be of sufficient quality, judged by its A₂₆₀/A₂₈₀ ratio. The isolated RNA was used immediately for subsequent cDNA synthesis using 1 µg of RNA, 15 µg/ml oligo(dT)₁₅, 1 mM dNTP's and 7000 U/ml M-MLV RNase H⁻ reverse transcriptase. A control without reverse transcriptase was always included.

PCR Analysis - PCR analysis was performed on an iCycler thermal cycler (BioRad, Venendaal, The Netherlands) using 20 U/ml AmpliTaq DNA polymerase (Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands), 2 µM of each primer (Life Technologies, Paisley, Scotland), 200 µM dNTP's and Perkin Elmer PCR buffer. Routinely, 32 cycles were run for K562 cells, and 40 cycles for all other cell-types, with 30 s denaturing at 95 °C, a 30 s annealing step, and 30 s extension at 72 °C, followed by a final extension of 7 min at 72 °C. Annealing temperatures, as well as other primer-specific conditions are indicated with the primers below. Two different primer-sets were used to detect cytochrome *b*₅₆₁ cDNA. All primers were designed to span over introns, so the amplification of unspliced genomic DNA would result in longer products than the amplification of cDNA. The primers used were b561/1 (fw GGAACGAAGCTAAACGCAC, rev GGGAAGGAGGAAGATGGTAG, T_{anneal} 56 °C, 1.5 mM MgCl₂, product 287 bp) and b561/srv (fw CACAGCACTGCCTTACTACG, rev

CTGGGAGCCGGGGCTATCTC, T_{anneal} 57.5 °C, 10% DMSO, 1.0 mM MgCl_2 , product 715 bp). The latter primers are identical to the ones used by Srivastava et al. (15). As a control for the quality of the cDNA, other primers were used for isotypes of the Na^+ , K^+ , -ATPase. These were β_1 (fw TGAACGAGGAGAGCGAAAG, rev AGTTGCCCAGTCCAAAATAC, T_{anneal} 58.5 °C, 2.0 mM MgCl_2 , product 281 bp) and β_2 (fw TGATGATTCGCCCCAAGAC, rev CGACATTCTACATTACCTCC, T_{anneal} 60.8 °C, 1.5 mM MgCl_2 , product 541 bp). After the PCR reaction, the products were analyzed on a 1.5% agarose gel containing 0.3 $\mu\text{g/ml}$ ethidiumbromide, and detected by transillumination using UV light.

Sequencing of PCR products was done by BaseClear (Leiden, The Netherlands).

Ghost preparation - White erythrocyte ghosts were prepared according to Weed et al. by hypotonic lysis of erythrocytes, obtained from the Bloodbank Leiden-Haaglanden (22). More densely packed membranes were obtained by ultrasonic treatment of the ghosts in a bath sonifier, and subsequent centrifugation. For blotting experiments, ghosts were prepared by hypotonic lysis of erythrocytes, followed by repeated washes of the membranes in 40 volumes of 5 mM potassium phosphate, pH 8.0, until white ghosts were obtained. Packed ghosts were dissolved in SDS sample buffer (0.1 M Tris-HCl pH 6.8, 10% glycerol, 5% β -mercaptoethanol, 3% SDS, bromophenol blue), homogenized with a syringe, and incubated for 1 hour at 37 °C. Samples were not boiled, as this could lead to aggregation of the cytochrome (23).

Cytochrome b_{561} positive control - Bovine cytochrome b_{561} was isolated from fresh bovine adrenal medullae. Fresh adrenals were collected from the local slaughterhouse, after which the medullae were removed, chopped, and ground using a mortar, under liquid nitrogen. A sample of ground medullae was dissolved in SDS sample buffer, and further treated as above. A human positive control was prepared similarly from membranes obtained from a human pheochromocytoma (kindly provided by Dr D.K. Apps, University of Edinburgh).

Western Blotting - Samples were separated by SDS-PAGE using a 10% separating gel in a Mini-V 8.10 vertical gel apparatus (Life Technologies, Breda, The Netherlands) at 175 V. After electrophoresis, the gel was equilibrated in transfer buffer (10% methanol, 0.19 M glycine, 25 mM Tris), and proteins were transferred to an Immobilon-P membrane (Millipore, Etten-Leur, The Netherlands) at 200 V for one hour. The membrane was blocked overnight in 3% BSA in Tris Buffered Saline/0.25% Tween-20 (TBS/Tween), and incubated with anti-bovine b_{561} rabbit serum, and subsequently with HRP-linked anti-rabbit IgG, with intermittent washing

in TBS/Tween. Both antibodies were diluted in blocking buffer. The blot was detected by ECL using New-RX X-ray film (Fuji Photo Film, Tilburg, The Netherlands). The rabbit sera, a gift from Dr. D.K. Apps, were produced by immunizing rabbits with purified bovine cytochrome b_{561} (24).

Spectral characterization - Absorption spectra were recorded on an Aminco DW-2a spectrophotometer at 77 K using a low-temperature accessory (J4-9603, Aminco) in special cuvettes with a 2 mm pathlength (25). Spectra were accumulated from 9 consecutive scans of one sample. Sonified erythrocyte membranes were routinely used, but the spectral properties of normal ghosts were found to be very similar. Samples were incubated in an argon-flushed, stirred glass cuvette, and were reduced by either 500 mM ascorbate or 5 mM sodium dithionite. The sensitivity to CO was studied by flushing dithionite-reduced samples with CO for several minutes. Samples were subsequently transferred to a low temperature cuvette, and immediately frozen in liquid nitrogen.

Results and Discussion.

To assess the possible participation of cytochrome b_{561} in electron transport across the erythrocyte membrane, the presence of this cytochrome in erythrocytes was studied by a number of techniques. Cytochromes can be characterized by their specific absorption bands. Figure 1 shows that spectra recorded at 77K gave at

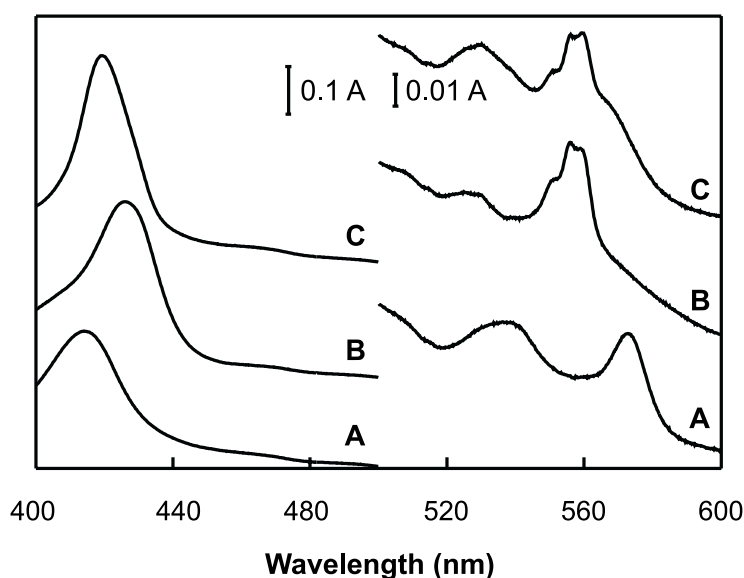


Figure 1. Absorption spectra of erythrocyte membranes. The spectra were recorded at 77K using membranes (5 mg/ml protein) that were oxidized (A), reduced with dithionite (B), or reduced with dithionite and treated with CO (C).

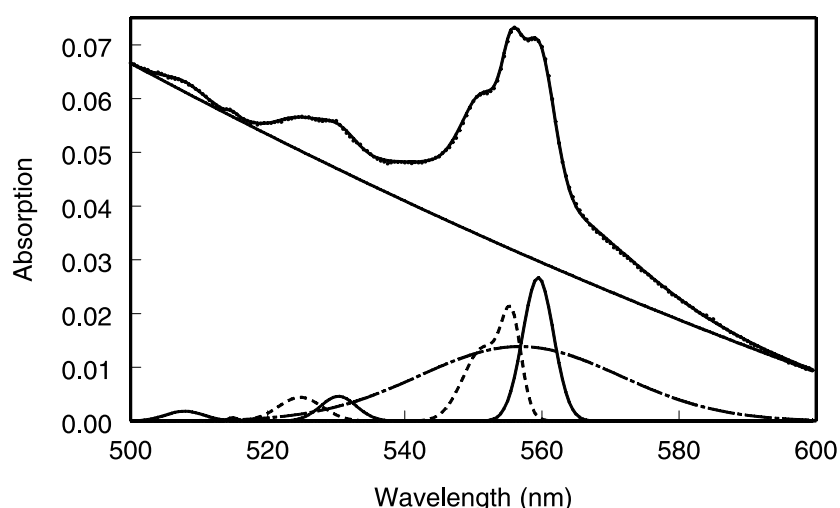


Figure 2. Deconvoluted absorption spectrum of reduced erythrocyte membranes.

In the upper part of the graph, the dots represent original data, while the solid line shows the composite simulation. The baseline is shown below the spectra, while the Gauss peaks are shown at the bottom. The peaks were assigned as follows: cytochrome b_5 (---), hemoglobin (-.-), and remaining peaks (—). For clarity, two peaks from cytochrome b_5 at 552 and 556 nm are combined.

least four absorption peaks under oxidized conditions and even more when reduced conditions were employed. This suggests that the membrane preparations contained more than one cytochrome. The spectra were further analyzed by spectral deconvolution, a mathematical technique in which a set of (Gauss) curves and a baseline are used to fit the experimental data. In this way, complex spectra can be deconvoluted to the peaks of their components, which can subsequently be identified on basis of literature data of known heme proteins. Figure 2 shows a typical result of the deconvolution and the subsequent peak assignments of a spectrum obtained from membranes reduced with dithionite. This figure demonstrates that the curve fitting procedure resulted in spectra similar to the experimental data. Table 1 summarizes the peaks observed in the spectra of figure 1. Comparison of data recorded under oxidized and reduced conditions indicates that the membrane preparations contained hemoglobin and cytochrome b_5 , as was already established in other studies (26). In addition, a cytochrome with absorption bands similar to that of cytochrome b_{561} (further referred to as cytochrome b_x) was found (27).

In subsequent experiments the CO sensitivity of the spectral components was measured. Addition of CO to dithionite-reduced samples induced changes in the spectrum, but the absorption peaks in the 550-560 nm region remained relatively unaffected (Fig. 1C). Moreover, it was found that the height of the peaks in this

Oxidized		Reduced		Reduced / CO	
Detected Peak	Origin (lit. value)	Detected Peak	Origin (lit. value)	Detected Peak	Origin (lit. value)
529	b_5 (530)	525	b_5 (525)	523	b_5 (525)
529	b_x	530	b_x	530	b_x (539)
540	Hb (540)	552	b_5 (552-3)	538	Hb (552-3)
564	b_5 (560)	556	b_5 (556-8)	552	b_5 (556-8)
564	b_x	557	Hb (555)	556	b_5
573	Hb (577)	559	b_x	560	b_x (569)
				565	Hb

Table 1: Assignment of absorption peaks. Absorption spectra of erythrocyte membranes, as shown in figure 1, were analyzed by mathematical deconvolution. Reduction was achieved by the addition of dithionite. The resulting peaks were compared to literature values of cytochrome b_5 and hemoglobin, known heme proteins of the erythrocyte (26, 28, 34, 35). Another component was found (indicated by b_x), resembling cytochrome b_{561} (27).

region was hardly affected by CO: the α -peaks of cytochrome b_5 remained at $122\% \pm 39$ compared to the reduced situation, and cytochrome b_x at $79\% \pm 7$ ($n=4$). As it is known that reduced hemoglobin binds CO, resulting in a shift of the spectral bands (28), while cytochromes b_5 and b_{561} do not, this confirmed our assignment of the peaks (Table 1).

If cytochrome b_x would be a component of an ascorbate-AFR transmembrane oxido-reductase, it can be expected that addition of ascorbate changes the oxidation state of the heme from oxidized to reduced. It was indeed observed that 500 μM ascorbate could reduce cytochrome b_x (data not shown), supporting the idea that it is involved in transmembrane electron transport. For both cytochrome b_x and b_5 , reduction by ascorbate amounted to 50-60% of the reduction achieved by dithionite. For cytochrome b_{561} , a comparable level of reduction has been described using 100 μM ascorbate, whereas 85% was reached with ascorbate levels up to 10 mM (16, 29).

Our data show that erythrocyte membranes contain a CO-insensitive cytochrome with an α -peak at 559 nm in its reduced state. This cytochrome can also be reduced by ascorbate. Its maximum absorption is at a wavelength lower than described for cytochrome b_{561} (27), but spectra recorded at 77 K can be shifted compared to spectra at room temperature. It is therefore conceivable that cytochrome b_{561} is a component of erythrocyte membranes, but a b -cytochrome with similar spectral characteristics could also explain our results. It has been

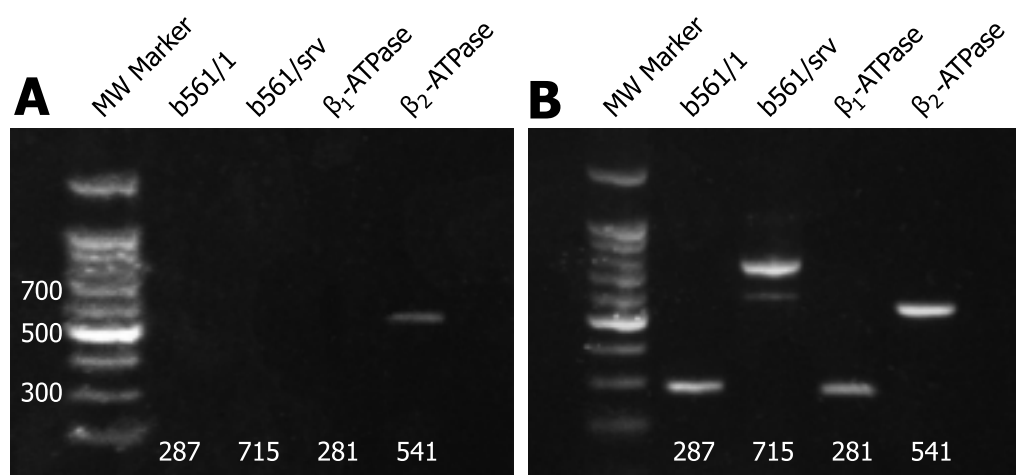


Figure 3. RT-PCR analysis. PCR-products prepared with different primers and cDNA obtained from reticulocytes (A) or K562 cells (B). The predicted lengths of products from the different primers are shown below each lane.

suggested that the heme protein with an absorption peak at 559 nm is cytochrome P420, a degradation product of cytochrome P450 (26). However, erythrocytes probably do not contain cytochrome P450, nor the P450-containing organelles (mitochondria and endoplasmic reticulum). Moreover, cytochrome P420 is expected to be CO sensitive, analogous to cytochrome P450. CO did not affect the 559-absorbing component, which excludes cytochrome P420 as a component of the red cell membrane.

To test the presence of cytochrome b_{561} in erythrocytes, a search for mRNA coding for this protein was performed. However, erythrocytes do not contain mRNA. If cytochrome b_{561} indeed is present in the erythrocyte membrane, it should be expressed during an earlier maturation stage in the hematopoietic pathway. It was therefore investigated whether cytochrome b_{561} mRNA was present in peripheral reticulocytes, the last erythrocyte progenitor cells still containing mRNA. For comparison, mRNA was isolated from human neutrophils, HL60 cells, and from K562 cells, a human erythroleukemic cell line that contains cytochrome b_{561} mRNA (15). cDNA was synthesized shortly after RNA isolation using oligo-dT₁₅ primers and PCR analysis of the cDNA was performed. In all cases, PCR reactions without template, as well as reactions with template from cDNA synthesized in the absence of reverse transcriptase, remained without products. It is therefore unlikely that genomic DNA or other contaminants were present.

Two different primer sets were used for detection of cytochrome b_{561} mRNA, but reticulocyte cDNA did not give a PCR product with either set (Fig. 3). cDNA from

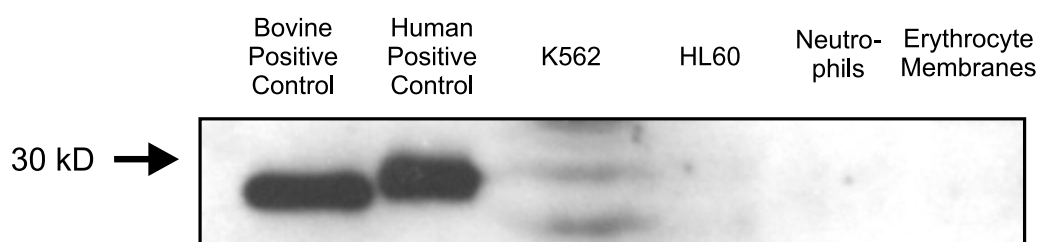


Figure 4. Western blot analysis. Extracts from K562 and HL60 cells, fresh human neutrophils, and erythrocyte membranes were separated by SDS-PAGE, blotted, and stained using an antiserum against bovine cytochrome b_{561} . As positive controls, extracts from bovine adrenal medulla, as well as extracts from human pheochromocytoma membranes were also included.

the K562 cell line, on the other hand, was positive for cytochrome b_{561} , showing that the PCR conditions were chosen appropriately. In addition, a PCR product obtained from $b_{561}/1$ primers and K562 cDNA was sequenced to verify its origin. The sequence of the PCR product was found to correspond to the expected section of the cytochrome b_{561} coding sequence (not shown), confirming the presence of cytochrome b_{561} mRNA in K562 cells. To check the quality of the reticulocyte mRNA, the presence of mRNA coding for another protein was investigated. It has been described that, at least in peripheral blood, reticulocytes are the only cells containing mRNA for the β_2 isotype of the Na^+, K^+ -ATP-ase, albeit in small amounts (30-32). Indeed, cDNA from reticulocytes produced a PCR product of the appropriate size when primers specific for the β_2 - Na^+, K^+ -ATPase were used (Fig. 3A). The band was not very strong, most likely due to the low abundance of the β_2 mRNA. Primers for the β_1 -isotype, which is expressed in e.g. leukocytes but not in reticulocytes, did not result in a product. In K562 cells both isotypes were expressed (Fig. 3B). Human neutrophils and HL60 cells were positive for both cytochrome b_{561} and the β_2 - Na^+, K^+ -ATPase, but not for the β_1 -isotype (results not shown). The results with HL60 cells are in contrast to earlier work, where cytochrome b_{561} was not found (15).

No cytochrome b_{561} mRNA could be detected in reticulocytes using RT-PCR. However, it is conceivable that the cytochrome would be transcribed at an earlier phase in erythrocyte development. In that case its mRNA could already have been degraded when the reticulocytes leave the bone marrow. Therefore, the presence of cytochrome b_{561} was studied at the protein level using Western blotting. As shown in figure 4, samples from human erythrocytes did not produce a band with a cytochrome b_{561} -specific antiserum. A positive control, i.e. cytochrome b_{561} from bovine adrenal medulla, gave a strong signal, as did a human pheochromocytoma

sample, indicating that the antisera cross-reacted with the human cytochrome b_{561} . Assuming that the erythrocyte component absorbing at 559 nm was indeed cytochrome b_{561} , the spectroscopic data indicated that at least 50 ng of the cytochrome should be present on the Western Blot (27). The human positive control contained 150 ng of pheochromocytoma membranes, in which up to 20% of the protein is cytochrome b_{561} (33). Thus, the amount of cytochrome from the erythrocyte sample loaded on the gel should have been well within the detection limit of the Western Blot. Nevertheless, no cytochrome b_{561} was detected by the anti-serum. K562 cells, which were positive for the cytochrome in the RT-PCR ((15) and Fig. 3), produced a faint band of the proper molecular weight (Fig. 4), showing that the b_{561} protein is present in these cells, albeit in small amounts. Cytochrome b_{561} could not be detected in extracts of HL60 or neutrophils, in spite of a positive RT-PCR result. Apparently, the levels are below the detection limit of Western blotting.

In conclusion, both RT-PCR and Western blotting experiments did not show the presence of cytochrome b_{561} in the erythrocyte. It is therefore concluded that the cytochrome is not the protein responsible for the ascorbate-dependent AFR reduction that was found in these cells. Apparently, another protein in the erythrocyte membrane is involved in this process. This protein may have strong similarities to cytochrome b_{561} , but homology searches on known sequences in genomic databases did not provide useful results. Alternative proteins could be expected to have transmembrane sections, and possibly high potential hemes as prosthetic groups. The unknown b -cytochrome found in our spectra could possibly play a role, but further investigations are necessary to elucidate its identity.

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